Natural Killer Cell Immune Surveillance of Lung Cancer

by

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Dedication

This dissertation is dedicated to my friends and family who have put up with me and my pursuit of science. I am sure I was rather tedious at times.

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Abstract

Microenvironments that tumor cells encounter are different between primary tumor, during metastasis, and at the site of metastasis, suggesting potential differences in immune surveillance of primary tumor and metastasis. Metastatic spread is the main contributing factor that results in 90% of solid tumor deaths. Studying and identifying targetable entities in the metastatic cascade would be exceptionally beneficial for patient treatment. These mechanisms may be cancer cell intrinsic or mediated by other cells of the body. Epithelial-mesenchymal transition (EMT) is a key reversible process in which cancer cells transition into highly motile and invasive cells for dissemination. EMT is considered a critical and initiating step in metastasis. After EMT, only a tiny proportion of cells successfully metastasize; supporting the notion of metastasis-specific immune surveillance. EMT involves extensive molecular reprogramming of cells conferring many clinically-relevant features to cancer cells and affect tumor cell interactions within and outside the tumor microenvironment.

Here, we will outline general principles of EMT and published works on the interactions with the immune system. This review will highlight the potential immunological consequences of EMT and the impact on tumor immune surveillance. Natural Killer (NK) cells are known to be the first line of defense against malignant cells and are critical for surveilling and eradicating tumors. Utilizing this knowledge of NK cells with *in vitro* and *in vivo* models of EMT, we investigated the role of NK cells in the context of metastasis-specific immune surveillance. Probing the molecular changes during EMT and known NK ligands revealed a skewing toward

activation of NK cells upon EMT. We identified two adhesion molecules on some tumor cell lines that drive the balance of NK activation *in vitro*, E-cadherin and Cell Adhesion Molecule 1 (CADM1). Deletion of the activating CADM1 ligand revealed an escape from NK-mediated immune surveillance. This loss correlates to two lung and one breast cancer patient cohorts showing reduced survival and metastatic spread in CADM1 low populations. We further determined the NK-specific surveillance and possible therapeutic relevance of CADM1 re-expression.

Our data demonstrated a metastasis-specific immune surveillance mechanism. Further, this mechanism was mediated by NK cells. Finally, we defined a novel function of a bone fide tumor suppressor, CADM1, in tumor immune escape. Taken together our data reveals a novel role for CADM1 and two avenues for therapeutic exploitation: EMT and boosting NK functions.

Chapter 1 General Introduction

Lung Cancer and Metastasis

Lung cancer is the leading cause of cancer deaths worldwide with approximately 1.5 million deaths per annum (Siegel et al., 2017). Lung cancer is typically associated with smoking tobacco products in the developed world, which is highly preventable. Though, in less developed regions of the globe lung cancer is also caused by the burning of organic fuels in the home. Exposure to carcinogens in the environment has also been associated with lung cancer. Exemplifying this, the second leading cause of lung cancer is radon exposure, a naturally occurring carcinogen (Darby et al., 2005; Krewski et al., 2006). Further, occupational carcinogens such as asbestos or air pollutants account for much of the non-smoking related lung cancer cases (van Loon et al., 1997).

The two major types of lung cancer are non-small cell lung cancer (NSCLC) and small-cell lung cancer, constituting approximately 85% and 15%, respectively, of patient diagnoses. Given the prevalence of NSCLC we will focus our studies here. NSCLC can be sub-stratified into three histological categories: adenocarcinoma, squamous cell carcinoma, and large-cell lung cancer. Smoking is more typically associated with squamous-cell and small-cell carcinoma. Adenocarcinoma is most associated with non-smokers (Herbst et al., 2008). Adenocarcinoma is the most common NSCLC at 40% of patients and, therefore, constitutes the bulk population of lung cancer patients. Adenocarcinomas arise from type II alveolar cells in the small airway

epithelium, are comparatively slow growing, and are found more towards the periphery of the lungs (Couraud et al., 2012).

The global five year survival rate of a patient diagnosed with lung cancer is a meager 17.7%. A critical factor owing to this dismal prognosis is due to metastatic spread (Weigelt et al., 2005). 80% of lung cancer patients have either regionally or distantly metastasized which leads to a precipitous drop in survival rate to just 4%. Metastasis is the primary cause of cancer-related mortality and can occur early through parallel progression along with the primary tumor or late after linear tumor progression (Klein, 2009).

The exact progressive steps are unknown in cancer, but what is clear is that metastasis is a highly inefficient process whereby millions of tumor cells are shed daily and only a few eventually colonize distant sites successfully (Gupta and Massague, 2006). The precise mechanism by which epithelial tumor cells disseminate from the primary tumor mass and enter either blood or lymphatic vessels to transit to distant sites is still under debate (Chambers et al., 2002; Lambert et al., 2017). Cancer cells may be forced into circulation by increasing pressures from the tumor mass and disrupted blood vessels that perfuse the tumor (Bockhorn et al., 2007). Additionally, cancer cells may crawl freely from the tumor mass and extravasate into the vessels and enter circulation. In order for this to occur, tumor cells must transition from a “stationary” epithelial-like cancer cell to a more motile phenotype.

There has been significant study into the concept of a plastic phenotypic switch that occurs to allow a epithelial-like cancer cell to migrate from the main tumor mass, circulate in the body, and then to colonize a distant site. Upon reaching the distant site an overt metastases that is histological similar to the primary tumor cell type of origin is typically formed which allows for

metastatic disease. There is a growing consensus that cancer cells adopt a developmental cellular program referred to as epithelial to mesenchymal transition (EMT). EMT was initially described during embryologic development whereby epithelial-like cells in the blastocyst migrate and form the various organ systems and structures (Kalluri and Weinberg, 2009). Given that all cells have this genetic blueprint for EMT, it has been suggested as a possible mechanism for how epithelial cancer cells utilize this cellular program and disseminate to distant sites.

Epithelial to Mesenchymal Transition as a Mechanism of Metastasis

In the context of cancer, EMT is a reversible cellular process by which stationary epithelial cancer cells trans-differentiate into highly motile and invasive mesenchymal-like cells, giving rise to disseminating or circulating tumor cells to initiate tumor metastasis (Bonnomet et al., 2010; Kalluri and Weinberg, 2009). The disseminated tumor cells, after reaching the target organ, undergo the reverse phenotypic conversion from mesenchymal-like back to epithelial-like through a process known as mesenchymal to epithelial transition (MET) (Thiery and Sleeman, 2006).

During EMT, cells downregulate the expression of multiple epithelial junctional proteins including E-cadherin (ECAD), an adherens junction protein, leading to the dissolution of cell-to-cell contacts, loss of apico-basal polarity, and acquire front-rear polarity (Kalluri and Weinberg, 2009; Thiery and Sleeman, 2006). Inhibition of ECAD expression is a hallmark of EMT and it is followed by the induction of proteins including N-cadherin, extracellular matrix components, and the enzymes that can degrade them. Cells also undergo a robust reorganization of actin-cytoskeletal architecture and a dramatic change in the cell shape. Together, the aforementioned key events result in a migratory and invasive capacity that defines the mesenchymal phenotype

(Kalluri and Weinberg, 2009; Thiery and Sleeman, 2006). The change in the epithelial and mesenchymal gene expression that occurs during EMT is regulated by multiple transcription factor families that include Snail, Twist, Zeb and bHLH (Lamouille et al., 2014). The transcription factor involved and its role not only depend on the cell and tissue type, but also on the signaling pathway that initiates EMT (Lamouille et al., 2014). The multifunctional cytokine, transforming growth factor beta (TGFβ), which is rich in tumor microenvironments and correlates with poor patient prognosis, has emerged as a potent inducer of EMT (Heldin et al., 2009; Massague, 2008).

Initiation and progression of TGFβ-induced EMT involves coordinated regulation of multiple signaling pathways by altering the expression or activation of their signaling components (Heldin et al., 2009; Massague, 2008). Several growth factors (EGF, HGF, FGF, IGF and PDGF) and developmental cytokines (Wnt, Notch and Hedgehog) are known to induce EMT as well (Lamouille et al., 2014). Similarly, inflammatory cytokines, including: TNF-α, IL-6, IL-1, and IL-8 in the tumor microenvironment have also been implicated in the induction of EMT (Jing et al., 2011; Lamouille et al., 2014). All of the non-TGFβ cytokines can induce EMT either through crosstalk with the TGFβ-dependent pathways or directly activate these pathways or induce the expression of EMT transcription factors (Jing et al., 2011). In addition to imparting a migratory and invasive capacity, EMT was shown to endow resistance to chemotherapy, radiation, confer stem cell-like properties, and is known to promote immunosuppressive mechanisms in the tumor microenvironment (Lamouille et al., 2014; Zavadil et al., 2008).

Recently, there have been two publications that have posited that EMT is not necessary for metastasis (Fischer et al., 2015; Zheng et al., 2015). Both studies have been rebutted by various lines of evidence as well (De Chiara and Crean, 2016; Li and Kang, 2016; Pattabiraman

and Weinberg, 2016). The Zheng et al. study focused on two EMT transcription factors, Twist1 and Snail1, and revealed that neither was necessary for metastatic progression of pancreatic cancer. However, EMT is not controlled by just two transcription factors and the metastatic progression could be the result of numerous other transcription factors implicated in EMT. The Fischer et al. study used a cell lineage tracing method to detect cells that have undergone EMT. Fibroblast-specific-protein 1 (Fsp1) was used to trace cells and showed that some metastases were negative for Fsp1. Suggesting that cells need not undergo EMT, even partially, to metastasize. Fsp1 has come under scrutiny for the uncertain specificity and sensitivity of this marker as it is only involved in some EMT programs, but not all. Neither study sufficiently proved that EMT did not occur, but revealed that EMT may take multiple forms depending on the molecular construction and type of the cancer cells.

Together, the abilities endowed by EMT may allow cancer cells to successfully navigate the highly inefficient process of metastasis and link EMT to major clinical aspects that are responsible for cancer-related mortality. In contrast, the process of MET is less characterized. While inhibition of TGFβ signaling or BMP-induced mir200 expression was shown to promote MET (Gregory et al., 2008), the precise molecular mechanisms involved are still not clear. Further study into the mechanism of MET will be of utmost importance to aid in the design of metastasis-specific therapies. A comprehensive understanding of all changes that occur during EMT, mechanisms that direct those changes, and pathways that mediate these events is needed. There are several extensive and outstanding reviews have been published previously (Kalluri and Weinberg, 2009; Lamouille et al., 2014) that discuss EMT and MET in greater depth that is outside the current scope.

Immune Surveillance in Lung Tumor Progression

During the process of metastasis, from EMT to MET, potentially allows for a window to target these disseminating cells. When these cells leave the immensely immunosuppressive tumor microenvironment they are subject to new pressures from the environment, especially the immune system. Mechanisms of tumor surveillance by the immune system have been intensively investigated in the primary tumor setting where the balance between pro- and anti-tumor mechanisms dictates the outcome of tumor initiation and growth (Grivennikov et al., 2010; Kitamura et al., 2015). The field of study that combines investigation into tumor intrinsic properties and immunological responses termed "tumor immunology" has been formulated in the past few decades. Though the core idea is not a new concept and the origins are rooted more than 100 years ago. Briefly, tumor immune surveillance was an idea initially posited by a Jewish German physician, Paul Ehrlich, in the first decade of the 1900s (Ehrlich, 1909). Therein, he predicted a process by which carcinogenesis is controlled by the body's defenses to insult in a similar manner to a foreign pathogen. A more formal delineation of cancer immune surveillance was proposed in the 1950s by Sir Macfarlane Burnet (Burnet, 1957). Here, he describes a process of targeting cancer in a similar manner to skin graft rejection from various strains of mice hinting at a more self-intrinsic method for targeting cancer. Currently, tumor immunology is defined as the interaction of cancer cells and the immune system.

The immune system is composed of two arms, the adaptive and the innate systems. Each arm has unique and overlapping functions that allow a robust response both to pathogenic insult and malignancy. The innate arm responds quickly and helps control initial infection while the adaptive arm begins to identify and expand a specific response. Adaptive immunity is composed of a T and B lymphocytes that can modulate their hypervariable receptors to specifically identify an epitope expressed on a target cell's major histocompatibility complex (MHC). Initiating this

response is an interaction by T cells with presented exogenous antigen by dendritic cells or B cells, among others, on MHCI or II. These antigens are typically from extra-cellular sources taken up by phagocytosis. This process allows T cells to identify the afflicted cells and subsequently clear infections (Murphy and Weaver, 2017).

Additionally, B cells can mature and create specific antibodies against foreign or perceived foreign antigens that can induce complement killing or antibody-dependent cell-mediated cytotoxicity. These antibodies are potent in controlling bacterial and viral infections. Further, antibodies can be directed against specific structural epitopes of proteins expressed on cell surfaces rather than MHCI loaded peptides as in the case with T cells. This allows for a robust and multifaceted approach to overcoming foreign pathogens. These concepts are the basis of the memory responses to specific epitopes re-encountered after a priming event or initial infection, and are the foundation of vaccine theory (Murphy and Weaver, 2017).

Recently, research has been focused on utilizing the adaptive immune response to fight malignancy in the body. The idea of blocking inhibitory receptors on T cells to reduce exhaustion and dysfunction in the tumor mass has been given the name “immune checkpoint inhibition”. The main receptor and ligand axis that has been studied is the PD1-PDL1/2 axis. It has been targeted and made it to the clinic as a new standard therapy in conjunction with traditional chemotherapies (Sun et al., 2018). Targeting these key regulators led to the successful development of check-point inhibitors that boost T-cell-mediated immunity against multiple tumor types, including non-small lung cancer (NSCLC) (Ott et al., 2013; Romano and Romero, 2015). These novel blocking antibodies are currently being used to explore efficacy against a wide range of cancers with over 1,000 clinical trials (Sun et al., 2018). There has been significant progress in helping a subset of patients, but even with the most efficacious of response there is

still 70% of patients that did not respond to these therapies. This work is still in its infancy and a more thorough understanding of what these drugs are capable of needs to be explored.

Contrary to the adaptive immune system's specific and variable response, the innate arm of the immune system is controlled by a germline encoded receptors that are not variable in their recognition of cognate ligands. A key difference is that the innate arm does not typically have a memory response to insults. Monocytes, macrophages, and other myeloid cells are some of the non-lymphocyte populations that can rapidly engage pathogens before the adaptive immune responses are needed. Dendritic cells bridge the gap between innate and adaptive arms by presenting the specific antigen to T or B cells for their identification of the specific target epitope. Further, there are innate lymphocytes that lack a hypervariable receptor that allows for exquisitely specific epitope recognition. Despite their lack of variable receptors Natural Killer (NK) lymphocytes utilize panoply of receptors to initiate cytotoxicity against target cells.

Both the innate and adaptive arms of the immune system have evolved to detect and eliminate foreign pathogens from the environment. This recognition can also involve cell intrinsic danger signals released by the infected or stressed cell. Similar danger signals can be expressed by malignantly transformed cells as well. Cancer cells are under significant stress as they have acquired novel mutations and cellular programs for various genetic, epigenetic, and/or environmental cues. Therefore, it is likely that cancer progression is shaped and constrained by the immune system and a break in surveillance leads to tumorigenesis and subsequent progressive disease if left unchecked.

Immune Editing of Cancer

As a cell begins to transform to a malignant cell, mutational burden and stress from transformation can induce danger signals to the immune system. Here, these early phase pre-malignant and malignant cells are eliminated by surveilling immune cells. These initial surveilling cells are typically from the innate arm of the immune systems as they do not require prior sensitization for activation. As the cancerous cells are killed or undergo apoptosis, phagocytic cells, typically macrophages or dendritic cells, uptake fragments and present antigens to the adaptive arm of the immune system in sentinel lymph nodes, known as tumor antigens if found to be specific by T or B cells. If the cancerous cells have created neo-antigens or antigens that are novel to the immune system, adaptive immunity may also play a role in control of tumor progression. This process can lead to inhibition of progression or even complete elimination.

If cancer cells evade the initial culling by the immune system, an equilibrium may be reached whereby the immune system controls cancer growth but does not eliminate the cancer cells. This is typically associated with the cancer cells either growing too rapidly and/or producing suppressive factors to "shutdown" the effector functions of the immune system. The modulation of immune cells in the tumor microenvironment to a less effective or tumor promoting phenotype can and frequently occurs. Examples of these switches are M1 to M2 phenotype of macrophages, inactivation of effector lymphocytes by tumor-derived suppressive factors (i.e. TGFβ), and recruitment of regulatory cell subtypes. Further, systemic effects can be mediated tumor-derived suppressive factors affecting the bone marrow compartment. These interactions can lead to the production of myeloid-derived suppressor cells that alter the immune landscape within the tumor microenvironment. The recruitment and creation of suppressive

elements within the tumor allows for equilibrium to exist between cancer progression and immune surveillance.

The equilibrium phase of tumor progression allows for further evolution of the tumor cells to evade immune targeting. Upon sufficient imbalance of immune surveillance, cancer cells have entered the escape phase of tumor progression. Herein, cancer cells have altered and modulated the local immune response to allow for evasion of immune surveillance mechanisms. An immunological tolerance state exists whereby immune cells are actively converted or stymied upon encounters with cancer cells in the primary tumor. The global tumor immunity is reduced and outgrowth can occur. **Figure 1-1** pictorially summarizes the three phases of cancer immune editing from elimination, equilibrium, and escape. Many concepts of tumor immunity are rooted in the control of primary tumors. The same concepts can and likely exist for transitioning or disseminating cells.

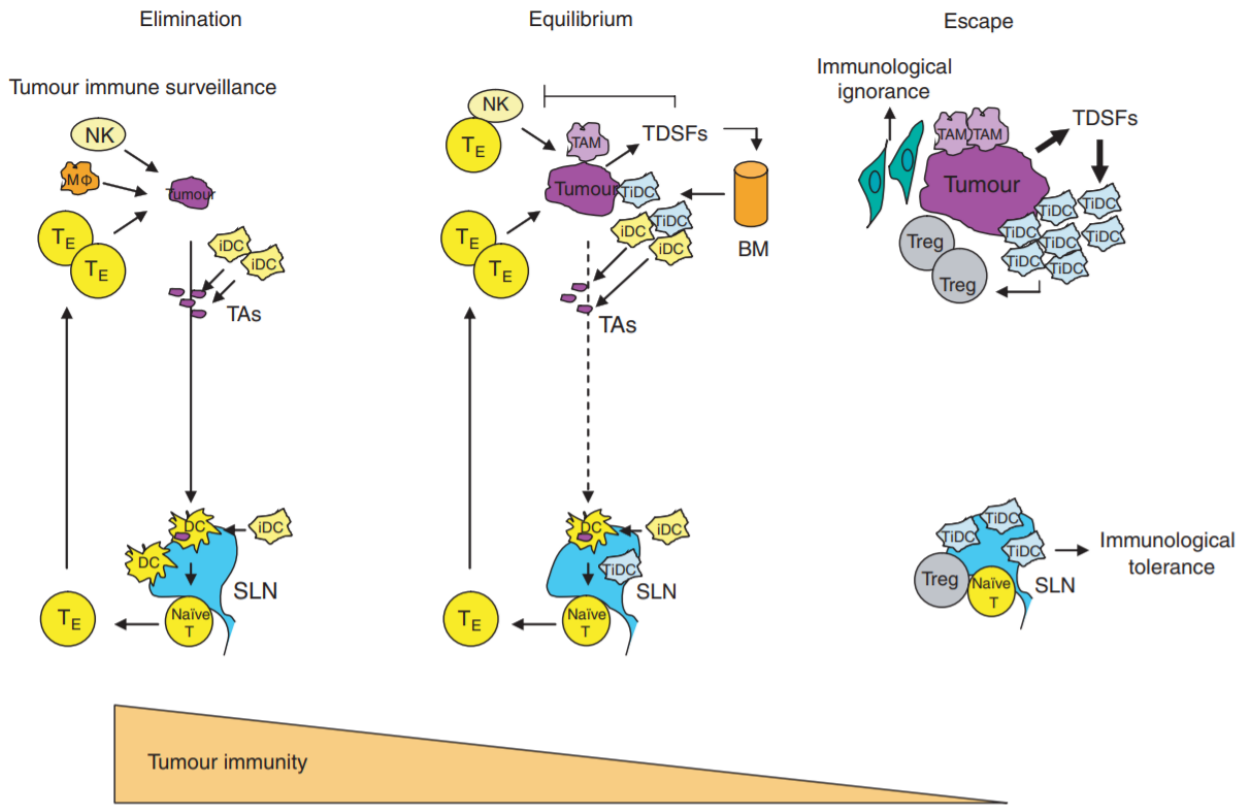

Figure 1-1 Overview of Immune Editing of Cancer

Adapted from (Kim et al., 2007) The three phases of cancer immune editing. Mϕ = Macrophage/Monocyte, T_E = Effector T cell, iDC = immature Dendritic Cell, TAs = Tumor Antigens, SLN = Sentinel Lymph Node, TAM = Tumor-associated Macrophage, TiDC = Tumor Inactivated Dendritic Cell, TDSFs = Tumor-derived Suppressive Factors, BM = Bone Marrow, Treg = Regulatory T cells

It is now increasingly clear that the microenvironment and the conditions tumor cells encounter in the primary tumor are disparate from what they encounter during metastasis. This suggests that mechanisms of immune surveillance in the primary tumor may also be different from those that regulate metastatic spread (Slaney et al., 2013) indicating the existence of metastasis-specific immune surveillance (Bidwell et al., 2012; Eyles et al., 2010; Hong et al., 2010; Olkhanud et al., 2009; Paolino et al., 2014; Slaney et al., 2013; Yang et al., 2008). As the primary tumor becomes less immunogenic due to selective pressures, a new wave of selective pressures likely awaits the cells as they leave. The selective pressures exerted upon the

metastasizing cells will likely be that much more potent as the transiting cells do not have a supportive niche to evolve and to evade the previously unknown surveillance mechanisms. This lends itself to the possibility of a potent metastasis-specific immune surveillance.

Metastasis-Specific Immune Surveillance

Studies thus far have focused on understanding how a tiny proportion of disseminating cells escape host surveillance and metastasize. Unfortunately, very little attention has been paid to the understanding of the mechanisms which successfully clear more than 99% of tumor cells. Though, it is the escape of these less than 1% of shed tumor cells that results in the lethal metastatic disease, but not exploiting the effective tumor clearance mechanisms that are already employed by the host may be a missed opportunity. Since EMT is critical for metastasis, exclusive focus on evasive or resistance mechanisms that cells acquire after EMT may have promoted an unintended bias; that cells undergoing EMT must be resistant to cancer immune surveillance.

On the contrary, it is equally feasible that metastatic cells, after EMT, are also vulnerable to host immune surveillance, as illustrated by the increased susceptibility to NK-mediated cytotoxicity (Lopez-Soto et al., 2013). In other words, when cancer cells exit the immunosuppressive primary tumor microenvironment, it is possible that they may pay a toll to metastasize by becoming more susceptible to host immune surveillance, and thus contribute to the inefficiency of metastasis. However, this concept needs further and more careful investigation. If proven, identifying the molecules and mechanisms that regulate these potential EMT-induced vulnerabilities may be critical for any metastasis-specific prevention strategies. Given the potential for the presence of metastasis-specific immune surveillance mechanisms, in

clinical trials, it may be important to somehow assess the efficacy of a given therapy on metastatic disease in tandem, even when there is no effect on the primary tumor. Reassessing patient data and analyzing treatment groups for metastatic spread post-trial may reveal some insight that would inform future clinical trials. These insights may suggest lengthier follow-up times to assess for metastatic events.

Regulation of EMT by Immune Microenvironments

Given the critical role of EMT in initiating and promoting tumor metastasis, we sought to understand the immunological consequences of EMT in tumor progression. To this end, we will review the impact of immune microenvironments on tumor cell EMT and then describe how cells undergoing EMT may interact with immune cells in the primary tumor and during metastasis. Finally, we will discuss the implications of molecular reprograming that occurs during EMT on metastasis-specific immune surveillance, tumor immunogenicity, and immunotherapies.

It is evident that inflammation plays a critical role at every stage of tumor development. Cancer cells produce various cytokines and chemokines that can recruit a diverse array of immune cells into the tumor including macrophages, neutrophils, dendritic cells, NK cells, mast cells, myeloid-derived suppressor cells, T and B lymphocytes, constituting tumor-associated inflammation (Grivennikov et al., 2010; Kitamura et al., 2015). Interactions of cancer cells with the immune microenvironment are critical for tumor progression beginning from tumor initiation to immune surveillance, promoting metastasis, and response to therapy (Elinav et al., 2013). All tumor-infiltrating immune cells are capable of producing multiple inflammatory mediators to modulate tumor progression.

Cancer cells, by engaging in a dynamic crosstalk with immune cells, exhibit EMT/MET plasticity to adapt to the changing microenvironment that they encounter in the primary tumor, during metastasis and at the distant site (**Table 1, Figure 1-3**) (Gao et al., 2012). These diverse interactions and the inflammatory mediators they produce, collectively and individually, can determine the course of tumor progression. In early stages, they can trigger neoplastic transformation by inducing genomic instability through production of DNA damaging agents. In later stages, they promote metastasis through multiple mechanisms including induction of EMT (Elinav et al., 2013).

Given the inflammatory environment that exists in early tumor formation, tumors have been described as wounds that do not heal (Kalluri and Zeisberg, 2006). Immune cells are recruited to sites of wounding for various roles to promote healing and elimination of any pathogens as well. Therefore, cancer and immune cell interactions have occurred since the initial stages of tumor growth. However, other stromal cells may already be present or recruited prior to immune cell infiltration. The following sections will describe some of the initial, mainly immune, cells that respond to tumors.

Fibroblasts

Fibroblasts, a traditional non-immune cell type, are a central cellular component of tumors and when associated with cancers are referred to as, cancer-associated fibroblasts (CAFs). CAFs provide signals to help growth and provide physical protection by surrounding cancer cells. Further, CAFs may aid in angiogenic recruitment to supply the tumor with blood flow (Kalluri and Zeisberg, 2006). Enhanced angiogenesis may also lead to increased metastatic spread. CAFs may also modulate resistance to chemotherapy and immune surveillance by producing extra-cellular matrix components. Growth factors are secreted by CAFs and modulate

the tumor landscape by enhancing cancer cell survival or altering immune responses. In a more cancer intrinsic manner, CAFs have been shown to alter the metabolism of cancer cells as well (Kalluri, 2016). Finally, CAFs can recruit immune cells to the tumor, namely, macrophages. Macrophages are part of the first wave of immune cells that are recruited to sites of wounding and, putatively, tumors.

Tumor-Associated Macrophages

Tumor-Associated Macrophages (TAMs) are one of the major components of the immune cell infiltrates observed in the tumor microenvironment. They are derived from inflammatory monocytes that are recruited largely by MCP-1/CCL2, chemokines (Lee et al., 2013; Pollard, 2009). TAMs are implicated in a multitude of tumor promoting functions including angiogenesis, immune suppression, and EMT (Gao et al., 2012; Kitamura et al., 2015). Genetic ablation of CSF1, a major lineage regulator of macrophages or deleting its direct effector, Ets2, results in macrophage depletion and marked reduction of metastasis in the *de novo* PyMT model of metastatic breast cancer (Lin et al., 2001; Zabuawala et al., 2010). In the same model, recruitment of TAMs results in a tumor microenvironment rich in TGFβ, a potent inducer of EMT along with mitogenic growth factors like PDGF and EGF. This leads to induction of EMT by TAMs, thus promoting metastasis (Kitamura et al., 2015; Pollard, 2009). TAMS are also a source for TNFα, IL-6, IL-1 and MMPs which are known to enhance TGFβ-induced EMT and subsequent invasion (Bonde et al., 2012).

Analysis of primary tumors from patients with NSCLC revealed a positive correlation between intratumoral macrophage densities, EMT markers, TGFβ levels, and tumor grade (Gao

et al., 2012). Even though the precise mechanisms by which TAMs mediate tumor progression *in vivo* are still unknown; they are implicated in every step of the metastatic cascade.

Following EMT, intravasation of cancer cells into vasculature is facilitated by perivascular TAMs (Wyckoff et al., 2007). During this process cancer cells secrete CSF1 for the recruitment of TAMs and TAMs in turn produce EGF and activate EGFR signaling in cancer cells. Together with other cytokines in the tumor microenvironment, TAMs induce an EMT-like phenotype with enhanced motility, invasion, and ECM degradation to promote intravasation of cancer cells (Condeelis and Pollard, 2006). Once in circulation, cancer cells have to survive anoikis, a form of cell death from lack of attachment. Many inflammatory mediators derived from both immune cells and cancer cells, including TNFα, IL-6, and epiregulin, can promote cancer cell survival by activating pathways such as NF-kB and Stat3 (Sullivan et al., 2009; Wu et al., 2009). In a mouse model of breast cancer, TAMs were shown to promote cancer cell survival by physically interacting during transit (Condeelis and Pollard, 2006).

Monocytes

Monocytes are a diverse set of cells consisting of several subtypes with distinct function in the tumor microenvironment. A monocyte subpopulation, named metastasis-associated monocytes (MAMs), was identified that preferentially migrate to metastatic sites rather than to primary tumors in breast and colorectal cancers (Qian et al., 2009). Similar to TAMS, MAMs are also derived from inflammatory monocytes, recruited by CCL2, and acquire a pro-metastatic phenotype. MAMs were shown to promote cancer cell extravasation and survival at metastatic sites. Importantly, neutralizing CCL2 blocked recruitment of MAMs and inhibited cancer cell extravasation (Qian and Pollard, 2010). Apart from the classical inflammatory monocytes which differentiate into TAMs or MAMs, a recent study also demonstrated a critical role for non-

classical “patrolling” monocytes (pMO) in tumor metastasis (Hanna et al., 2015). This study showed that pMOs accumulate in the microvasculature of lung and inhibit lung metastasis in multiple mouse models. pMOs reduce metastasis by interacting with cancer cells in the vessels and later recruit and activate NK cells (Hanna et al., 2015). Unlike TAMs which promote EMT and metastasis, it is of interest to understand the effects of MAMs and pMOs on cancer cells. For instance, it would be fascinating to see if MAMs promote MET at metastatic sites to facilitate successful colonization by cancer cells.

Neutrophils

Studies thus far demonstrate both pro- and anti-metastatic effects of neutrophils during tumor progression. Depletion of neutrophils promoted lung metastasis in a mouse model of breast cancer (Granot et al., 2011). Consistent with this observation, neutrophils isolated from tumor bearing mice showed cytotoxicity against cancer cells *in vitro* and adoptive transfer of these neutrophils blocked experimental lung metastasis (Granot et al., 2011). Neutrophils also produce unique structures called neutrophil extracellular traps (NETs) which are composed of extruded DNA and antimicrobial proteins. After surgical stress or infection, cancer cells have been shown to become trapped in NETs which formed in liver and lung capillaries promoting the development of micrometastases (Cools-Lartigue et al., 2013; Tohme et al., 2016).

On the other hand, cancer cells were also shown to recruit neutrophils through CXCL15 or HMGB1 secretion (Fridlender et al., 2009). Recruited neutrophils were implicated in enhancing angiogenesis, intravasation of cancer cells, and suppression of cytotoxic CD8 T lymphocytes; thus promoting metastasis (Coffelt et al., 2015; Kitamura et al., 2015). Interestingly, TGFβ has been shown to induce a switch from anti-metastatic to a pro-metastatic

phenotype in neutrophils in a mouse model of mesothelioma (Fridlender et al., 2009). Therefore, it is possible that the pro-metastatic functions of neutrophils are regulated by specific environmental factors in a similar manner to TAMs; and like TAMs, neutrophils may also modulate EMT, at least in the context where they are known to promote metastasis.

Platelets

Also known as thrombocytes are small, enucleated cellular structures and are second most abundant in circulation after erythrocytes (Goubran et al., 2014). The primary role of these cells is to stop bleeding (hemostasis) after tissue or vascular injury (Goubran et al., 2014). Increased platelet numbers have been associated with decreased patient survival in a number of tumor types including, breast, lung, pancreatic, and brain, suggesting a role for platelets in tumor progression (van Es et al., 2014). In circulation, platelets form platelet-cancer cell aggregates to aid and shield migrating cancer cells by multiple mechanisms and promote metastasis. In colon and breast cancer, platelets promote extravasation of cancer cells by inducing EMT, through direct contact and release of TGFβ (Labelle et al., 2011). Platelet-specific ablation of TGFβ production or cancer cell-specific inhibition of NF-kB activation protected mice from tumor metastasis (Labelle et al., 2011). In melanoma, platelet-derived ATP was shown to activate a purinergic receptor, P2Y, on endothelial cells to increase vascular permeability and promote cancer cell extravasation (Schumacher et al., 2013). In this study, genetic ablation of P2Y suppressed metastasis (Schumacher et al., 2013). Formation of platelet-cancer cell aggregates may also protect circulating cancer cells from NK and T cell-mediated immune surveillance (Palumbo et al., 2005).

In addition, platelet-derived cytokines, including PDGF, VEGF, and TGFβ, can promote cancer cell survival, angiogenesis, and EMT in the primary tumor microenvironment and promote metastasis (Goubran et al., 2014). Given the dynamic molecular changes that occur during EMT, it is reasonable to expect potential differences between epithelial and mesenchymal phenotypes in their ability to interact with platelets. Investigating these differences may help in the targeting of platelet-cancer cell interactions for metastatic control.

Myeloid-Derived Suppressor Cells (MDSCs)

Abnormal differentiation of the myeloid compartment in tumor-bearing mice and cancer patient's results in the accumulation of immature immunosuppressive myeloid cells called MDSCs, reflecting their origin and function (Condamine et al., 2015). MDSCs contribute to tumor progression by a variety of immune suppression-dependent and independent mechanisms. MDSCs are known to produce a plethora of soluble factors, including MMPs, CXCL12, CXCL5, VEGF, bFGF, HGF, and TGFβ, to promote angiogenesis, cancer cell invasion, and metastasis. The clinical relevance of MDSCs was demonstrated in multiple cancers where the number of circulating MDSCs in patients was positively correlated with advanced disease stage and metastasis (Diaz-Montero et al., 2009; Sun et al., 2012; Wang et al., 2013; Zhang et al., 2013). In a spontaneous mouse model of melanoma, MDSCs recruited to the tumor site produced HGF and TGFβ to induce EMT in melanoma cells. Depletion of MDSCs suppressed melanoma metastasis by inhibiting cancer cell EMT (Cui et al., 2013).

MDSCs are also known to promote metastasis by inducing cancer cell stemness in ovarian cancer (Toh et al., 2011). Intriguingly, MDSCs are implicated in the formation of pre-metastatic niches where MDSCs reach the niche before the cancer cells and condition it to

promote cancer cell seeding by secretion of immunosuppressive factors such as S100A8/A9, bFGF, IL-10, and IL-4. Once cancer cells reach this metastatic niche, MDSCs are implicated in promoting MET in cancer cells by secreting versican (Kim et al., 2009). There remains a lack of clear understanding of the precise mechanism by which MDSCs are recruited to the pre-metastatic niche.

Indirect Mechanisms

Aside from the aforementioned direct effects on cancer cells resulting from cell-to-cell interactions with immune cells, the inflammatory cytokines produced by all immune cell types can modulate EMT through indirect mechanisms. The transcription factor Snail, an important regulator of ECAD expression during EMT, is protected from degradation in response to TNFα signaling. Thus, stabilization of Snail aids in completing the process of EMT and promoting cancer cell migration and metastasis (Wu et al., 2009). Similarly, other EMT transcription factors like Twist and Kiss are regulated by pro-inflammatory cytokines (Yu et al., 2009) including complement component C3a (Cho et al., 2016). Activation of Stat3 was implicated in Twist induction and NF-kB-mediated induction was shown for Twist and Kiss expression (Wu et al., 2009; Yu et al., 2009). EMT-induced cancer cell invasion requires extensive proteolysis of the extracellular matrix (ECM). In addition to cancer cells, inflammatory immune cells are also important source for ECM degrading proteases including MMP2 and MMP9. Again, cytokines like TNFα, IL-6 and IL-1 are implicated in the induction of these proteases. After EMT and metastatic cells enter circulation, these same cytokines promote the survival of tumor cells in circulation through activation of NF-kB and Stat3-mediated survival pathways (Grivennikov et al., 2010; Wu et al., 2009; Yu et al., 2009).

Impact of EMT on Cancer and Immune Cell Interactions

Another critically important aspect of the tumor microenvironment crosstalk is the ability of cancer cells to modulate immune responses within the tumor. The most common theme in these interactions has been that cancer cells interfere with the anti-tumor responses by secreting soluble mediators that block the effector functions of the involved immune cells and reprogram them into cells of a regulatory phenotype. Robust morphological and molecular changes that occur during EMT support the idea that cells undergoing EMT have the potential to modulate the function and phenotypes of both innate and adaptive immune cells in the tumor microenvironment (**Table 1** and **Figure 1-5**). However, only a few studies have examined the impact of EMT on the interactions between cancer and immune cells (Chockley and Keshamouni, 2016).

Impact on Tumor-Associated Macrophages

After the recruitment of macrophages into tumor microenvironment, the reciprocal interaction between macrophages and cancer cells involves modulation of macrophage phenotype by cells undergoing EMT. Studies have shown that cancer cells can skew macrophages towards an M2 phenotype that is associated with TAMs, through production of various factors such as IL-4, GM-CSF, and TGFβ (Qian and Pollard, 2010). Although, precise mechanisms by which macrophages acquire the tumor promoting TAM phenotype are not clear, recent studies, including ours, identified a role for TLR signaling. We demonstrated that tumor cell-derived TGFβ induces the expression of IRAK-M, a negative regulator of TLR signaling, in macrophages promoting an M2 phenotype (Standiford et al., 2011). Genetic ablation of IRAK-M

in mice inhibited tumor growth by promoting an M1 like phenotype in TAMs (Standiford et al., 2011).

In another example, screening for cancer cell-derived factors that promoted macrophage activation identified the extra-cellular matrix component versican in mouse lung cancer cells (Kim et al., 2009). Versican is also upregulated in many human tumors. This study demonstrated that versican activated macrophages through TLR2, induced IL-6, and TNF-α to generate a microenvironment that facilitates metastatic outgrowth of Lewis Lung Carcinoma cells in mice (Kim et al., 2009). A similar skewing of neutrophils to a more tolerogenic phenotype by cancer cells was also reported in the tumor microenvironment (Fridlender et al., 2009). However, in all the above studies differential impact of epithelial versus mesenchymal phenotypes was not assessed. Utilizing a Snail1-overexpression model, EMT cells were shown to induce differentiation of immature DCs into regulatory DCs, with low MHC class II expression (Kudo-Saito et al., 2009).

EMT and Cytotoxic T Lymphocyte Functions

To date, unlike innate immune cells, there is no evidence that T and B cells can directly modulate tumor cell phenotype, including induction of EMT, despite their contribution to the overall tumor progression. On the contrary, cells undergoing EMT were shown to induce the activation of immunosuppressive T-reg cells. Utilizing both TGFβ-induced as well as Snail1-overexpression models of EMT in melanoma cells, TSP1 (thrombospondin-1) produced during EMT was implicated in the induction of FOXP3 expression in CD4+ T cells (Kudo-Saito et al., 2009). Inhibition of Snail1 or neutralizing TSP1 was sufficient to restore T-cell infiltration and induction of anti-tumor immune responses in the B16-F10 melanoma tumors (Kudo-Saito et al.,

2009). In MCF-7 human breast cancer cells, acquisition of EMT phenotype was associated with the inhibition of cytotoxic T lymphocyte (CTL)-mediated lysis (Akalay et al., 2013). This inhibition in CTL-mediated lysis was attributed to the dysfunctional immunologic synapse between CTLs and cancer cells, and to the induction of autophagy in cancer cells. Interestingly, inhibition of autophagy in cancer cells restored susceptibility to CTL-mediated cytotoxicity (Akalay et al., 2013). This is consistent with the fact that the extensive actin cytoskeletal remodeling that occurs during EMT is also critical for the formation of immunological synapses (Abouzahr et al., 2006).

These data suggests that in addition to the recognition of a tumor cell, the formation of a successful immunological synapse is also critical for host immune surveillance. The observed differences in the ability of epithelial and mesenchymal-like cells to form immunological synapses may also contribute to metastasis-specific immune surveillance. In a unique mouse model of melanoma in which tumor cells disseminate early even before the primary tumor is detectable, disseminated tumor cells were kept dormant at distant sites, in part, by cytostatic CD8+ T cells. Depletion of these cells allowed metastatic outgrowth, demonstrating immune control of metastasis (Eyles et al., 2010).

A more recent study demonstrated an important molecular link between EMT and CTL dysfunction. This study provided evidence that microRNA-200 (miR-200), a suppressor of EMT, targets PD-L1, which is a ligand for the CTL check-point receptor PD-1 (Chen et al., 2014). Transcription factor ZEB1, an EMT activator, induces PD-L1 expression on tumor cells by blocking the miR-200-mediated suppression of PD-L1, resulting in the suppression of CTL function and promotion of metastasis. These findings suggest that the EMT phenotype may serve

as a biomarker to identify subgroups of patients who may respond to check-point inhibitors such as PD-L1 and CTLA4 antagonists.

Regulation of Complement-Mediated Cytotoxicity

The complement pathway is recognized as a first non-cellular line of defense in host immune surveillance against non-self-microbial and tumor cells (Walport, 2001). The deposition and activation of complement component proteins in tumor tissues, coupled with increased expression of inhibitory complement regulatory proteins on tumor cells illustrate the importance of complement pathway in host immune surveillance against cancer (Donin et al., 2003; Fishelson et al., 2003; Jurianz et al., 1999; Varsano et al., 1998). In a recent study, we observed resistance to complement-dependent cytotoxicity and induction of CD59 expression after TGFβ-induced EMT in lung cancer cells. CD59 is a potent inhibitor of membrane attack complex that mediates complement-dependent cytotoxicity (CDC). Inhibition of CD59 expression restored susceptibility to CDC of cells that have undergone EMT *in vitro* and blocked experimental metastasis by these cells (Goswami et al., 2016).

Conversely, complement activating components have also been implicated in promoting tumor progression. For example, complement component C3a was shown to trigger EMT in ovarian cancer cells through the induction of an EMT transcription factor, Twist (Cho et al., 2016). One possibility for such paradoxical effects might be that EMT renders cancer cells resistant to complement-mediated cytotoxicity, after which the activating components of the complement might promote tumor progression by sustaining EMT. The co-opting of complement components for tumor survival highlights the evolution of cancer upon selective pressures of the

immune system similar to resistance of bacteria to antibiotics. If cancer cells escape the complement system then they must now evade the first cellular line of defense: NK cells.

Natural Killer Cells

NK cells were first described for their ability to detect cancer cells and eradicate them without prior sensitization (Jondal and Pross, 1975; Kiessling et al., 1975; Pross and Jondal, 1975; Spits et al., 2016; West et al., 1977). NK cells utilize a panoply of stochastically-expressed receptors that identify stressed or virally-infected cells. Additionally, it is theorized that NK cells distinguish self from non-self via MHCI recognition (Vivier et al., 2012). Consequently, NK cells are inhibited when interacting with a cell that is expressing self MHCI and would target a cell lacking or expressing non-self MHCI. MHCI is expressed on all nucleated cells of the body and, typically, express self-peptides from inside the cell. The rapid response enacted by NK cells places them as one of the first lines of defense against virally infected or malignant cells.

How NK cells selective target cells, has slowly been elucidated over the years. Unlike T or B cells, NK cells sense both activating and inhibitory ligands and the culmination of signaling tips the balance of the response (Long et al., 2013). Internal adaptor proteins transduce the signals received and are shared among many receptors. However, the ligands recognized by NK cells are not necessarily only associated with stress or viral infection. Proteins that are not typically exposed to circulating or surveilling NK cells can trigger responses as well. As an example, a disrupted epithelial cell barrier may expose intercellular junctional proteins that indicate some damage may have occurred. New ligands are being discovered and attributed to various responses mediated by NK cells (Barrow et al., 2018). An infographic of relevant

receptor and adaptor proteins is shown below (**Figure 1-2**). Herein, the diversity of receptors and the relatively restricted number of adaptor molecules for signal transduction is shown.

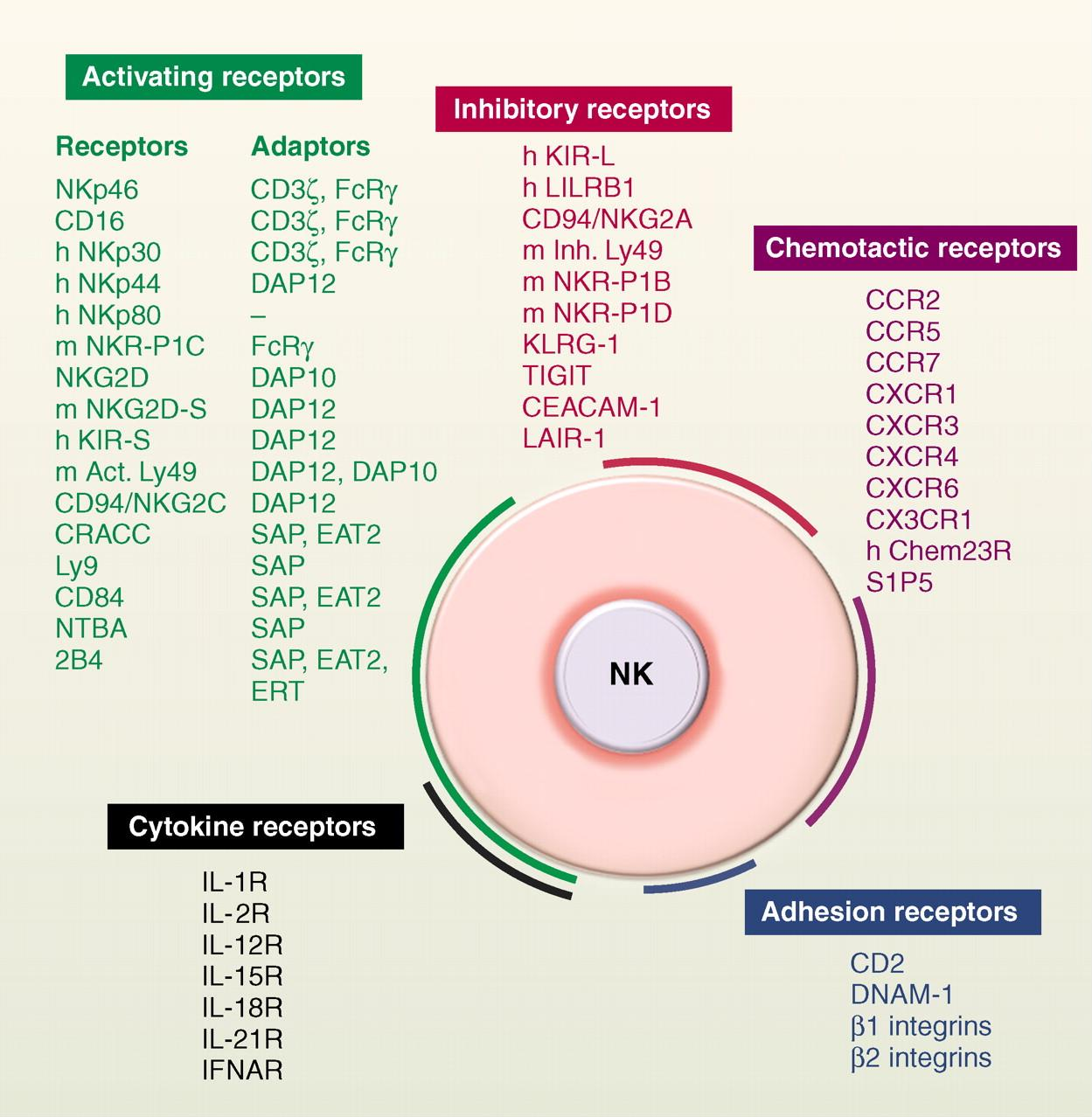

Figure 1-2 Natural Killer Receptors

Figure taken from (Vivier et al., 2011) A general overview of the various receptors utilized by human and mouse NK cells. “h” designates human and “m” designates murine.

Receptor/Ligand Pairs Used by NK Cells

Some NK cell receptors are conserved across species and can interact with ligands from xenogeneic sources. The cross reactivity that can occur between species highlights the evolved nature of the immune system. The innate arm is evolutionarily older than the adaptive immune system (Cooper and Herrin, 2010). The conserved nature of some receptors and their ligands lends itself to the ubiquitous existence of cancer as well. It is unsurprising that similar mechanisms are employed across species to control malignancy. This homology can allow for more precise modeling and elucidation of molecular mechanisms when using murine or primate models with human cancers. **Figure 1-3** shows selected ligands and their respective reactivity and cross-reactivity between mouse and human. NKG2D is one of the most widely studied activating receptors in mouse and humans. Intriguingly, murine and human NKG2D ligands to not cross activate the receptor despite the similarities in the receptor.

NK Ligand Name	NK Receptor Name	Receptor Type
E-Cadherin	KLRG1	Inhibitory
HLA Class I	LIR-1	Inhibitory
PVR	TIGIT	Inhibitory
PVR	DNAM-1	Activating
PVR	TACTILE	Activating
CADM1	CRTAM	Activating
CD48	2B4	Activating
ULBP2	NKG2D	Activating
ULBP4	NKG2D	Activating
MicA/B	NKG2D	Activating
H60, RAE, MULT1	NKG2D	Activating

Specificity: Human Both Mouse

Figure 1-3 Comparison of Human and Mouse Natural Killer Receptor Ligand Pairs

Receptor type is defined by human intracellular ITIM or ITAM domains or experimentally determined responses. Only a few selected ligand receptor pairs are shown.

Furthermore, some receptors in mouse exert opposite roles in humans and vice versa (Blake et al., 2016a; Fuchs et al., 2004). Conversely, there are some receptor and ligand pairs that are cross-reactive, specifically the nectin and nectin-like adhesion molecules. The duality of some NK receptors, being cross-reactive or performing opposite roles, adds to the complexity of NK cell biology and has hindered their study over the years.

Adhesion Molecule Signaling of Nectins and Nectin-like Proteins

One of the first nectin-like proteins identified to play an important role in NK function was the poliovirus receptor, CD155. CD155 binds with three distinct receptors on NK cells; CD96, TIGIT, and DNAM-1(also known as CD226) (Fuchs and Colonna, 2006; Martinet and

Smyth, 2015; Stanietsky and Mandelboim, 2010). CD96 and TIGIT have been shown to be inhibitory receptors, whereas DNAM-1 is an activating receptor (Chan et al., 2014; de Andrade et al., 2014). All three receptors can bind with various other members of the nectin and nectin-like family, potentially modulating previously observed responses. (**Figure 1-4**) shows the exceptional promiscuity of the nectin and nectin-like family of proteins.

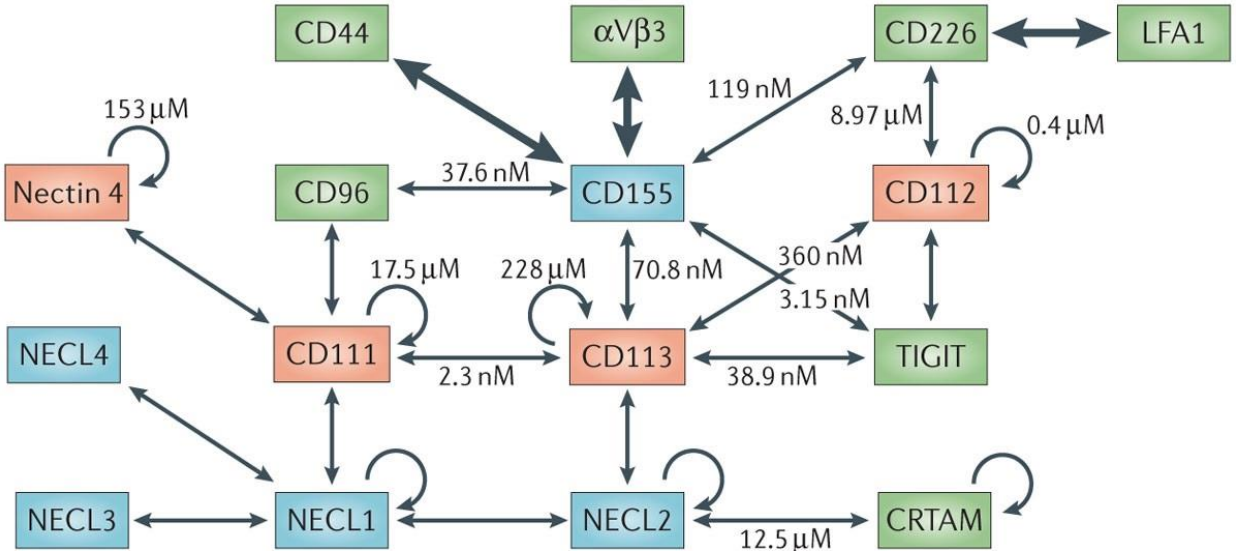

Figure 1-4 Nectin and Nectin-Like Molecule Binding Patterns

Figure taken from (Martinet and Smyth, 2015) Blue boxes represent nectin-like proteins, red boxes are nectin proteins, and green boxes are known receptors. Dissociation constants are shown for various interactions indicative of binding affinity.

Recently, blockade of TIGIT was shown to prevent NK cell exhaustion and mediate tumor immune surveillance (Zhang et al., 2018). This occurred in conjunction with concomitant immunotherapy treatment, anti-PDL-1, and was shown to be NK-dependent in both cases. DNAM-1 has been the most thoroughly studied in the context of NK cell tumor cytotoxicity (Bottino et al., 2003; Chan et al., 2014; de Andrade et al., 2014; Gilfillan et al., 2008; Guillerey et al., 2015) Herein, it is shown that when NK cells lack DNAM-1, tumor growth is enhanced and metastatic burden is increased. Intriguingly, all of these studies are based on the idea that

these receptors are binding with CD155, but there is such significant overlap in alternate ligand binding that it is difficult to fully elucidate the mechanisms involved. Despite the significant recent advances in our understanding nectin and nectin-like signaling in the context of cancer immune surveillance, CADM1 (also known as NECL2) and CRTAM have been thoroughly overlooked, especially in the context of NK cells.

There has been some work exploring the role of CADM1 interaction with T cells. One study suggested a role for CD8 T cells in the control of breast cancer metastasis. Here, breast cancer cell lines were modified to express CADM1 and both tumor growth and metastasis was affected. However, there were critical errors in design and analysis making the stated conclusions unsupported by the data presented (Faraji et al., 2012). Furthermore, a subset of γδ T cells upon CRTAM and CADM1 interactions triggers cell death (Catros et al., 2014; Fournier et al., 2010). This indicates a potential immunological benefit to retaining CADM1. Again, this highlights the precarious balance that cancer cells have to achieve to evade the immune system.

Additionally, a recent profiling of human lung adenocarcinoma patients revealed that NK cells were the most reduced cell type in the primary tumor compared to circulating blood or normal lung tissue (Lavin et al., 2017). This evidence supports the notion that NK cells are critical in controlling tumor initiation and growth. Further, rates of tumor initiation in murine models are unaffected by the adaptive immune system (Outzen et al., 1975). Though, it is well studied that after initiation, tumor progression can be affected by the adaptive immune system both positively and negatively.

In summary, cancer cells are at a crossroads of how to evade the two arms of the immune system. Given that cancer cells arise from the body and are not truly foreign, the manner in

which the adaptive immune system works to stop foreign pathogens selects for non-self-reacting T and B cells specifically. Failure of non-self-selection leads to autoimmunity. The entire CD8 T cell adaptive arm can be evaded by downregulating MHCI, but this leaves the innate arm, specifically NK cells, ready to attack the now "foreign" cancer cell. Cancer cells must then employ myriad of mechanisms to modulate inhibitory ligands and activating ligands to evade both arms and to continue to grow. Due to the unstable nature of cancer genomes by truncating or adding genetic information, these cells readily respond to environmental pressures. Tumor progression, survival, and metastatic spread are critically dictated by EMT and therefore offer an opportunity to target these transitioning cancer cells to potentially halt metastasis.

Potential Clinical Implications

Each of the studies described above has been carried out in isolation and in different models. However, the effects of different immune cell types in modulating various aspects of tumor progression are similar. As many of these immune cells work together in other contexts, it is likely that they also work together during primary tumor growth and metastatic seeding. Initiation and progression of EMT involves a robust reprogramming of gene expression, changes in signaling and metabolic pathways, and reorganization of the cytoskeleton. Given the wide spectrum of changes that occur during EMT, it is reasonable to speculate that EMT can have a broad range of consequences for cancer cells, host immune surveillance, and the efficacy of immune therapies as discussed below.

Tumor Immunogenicity

Many conventional therapies, including chemotherapy, radiation therapy, and targeted therapies have been shown to rely on the induction of anti-tumor immune responses for their

optimum efficacy (Zitvogel et al., 2011). However, the triggering of an anti-tumor immune response depends on the immunogenicity of a tumor. Immunogenicity is dictated by the cancer cell antigenicity and the multitude of other factors produced in the tumor microenvironment (Blankenstein et al., 2012). Several mechanisms, including genetic and epigenetic changes are known to regulate both antigen expression and antigen presentation, which are two major factors that regulate tumor immunogenicity. What is not fully considered in this process is the plasticity of cancer cells to undergo EMT. It is now well established that induction of EMT involves robust modulation of cell surface proteins, isoform switching by alternative splicing, immune modulatory cytokine secretion, and actin cytoskeletal remodeling (Lamouille et al., 2014; Philippar et al., 2008). Each of the changes occurring during EMT is capable of generating neo-epitopes and modulates their presentation. As a result, EMT may alter tumor immunogenicity at a much faster rate than genetic effects, which are inherently slower. Cataloging molecular changes during EMT, particularly ones that have the potential to modulate immunogenicity may identify novel antigens to design primary tumor- and metastasis-specific immunotherapeutic strategies.

EMT Confers Susceptibility to NK Cell-Mediated Cytotoxicity?

An epidemiological study showed that low NK cell activity in blood correlates with high incidence of malignancies, suggesting a critical role for NK cells in host's immune surveillance against cancer (Imai et al., 2000). Like T cells, despite their presence in the tumors there is little evidence that NK cells actively contribute to tumor progression, including induction of EMT. Consistently, tumor infiltration of NK cells was mostly associated with either better patient prognosis or had no influence at all. On the contrary, the immunosuppressive tumor

microenvironment, which may also include cells undergoing EMT, renders tumor-infiltrating NK cells hyporesponsive with low cytotoxic activity (Platonova et al., 2011).

The other major obstacle for NK-mediated immune surveillance is their limited access to cancer cells in the tumor bed. Multiple studies have shown that NK cells, when present, are preferentially localized to tumor stroma with little or no direct contact with cancer cells (Carrega et al., 2008; Chow et al., 2012; Halama et al., 2011; Platonova et al., 2011). Further, highlighting the restricted nature of NK cells and lung cancer, a recent study revealed that NK cells were the most reduced cell population in primary lung tumors compared to normal lung or blood (Lavin et al., 2017). Emerging data suggests that circulating NK cells are potent killers of cancer cells compared to organ-specific (Halfteck et al., 2009) or tumor-infiltrating NK cells (Platonova et al., 2011). In agreement with this hypothesis, circulating NK cells were shown to be crucial for prevention of metastasis (Sathe et al., 2014), but the mechanisms involved are not clear.

Recently, acquisition of mesenchymal-like phenotype has been shown to increase the expression of NKG2D ligands, a major class of NK cell activators, rendering cells undergoing EMT more susceptible to NK-mediated cytotoxicity (Lopez-Soto et al., 2013). Consistent with this finding, we observed a similar increase in susceptibility to NK-mediated killing in lung cancer cells after TGFβ-induced EMT. However, the mechanism was independent of the NKG2D receptor (Chockley et al., 2018). This suggests that EMT cells may become susceptible through multiple mechanisms. Together, the above observations indicate that cells undergoing EMT, while contributing to the immunosuppressive microenvironment that inhibits NK-mediated immune surveillance, when in circulation they become more susceptible to NK-mediated cytotoxicity. This is consistent with the notion of metastasis-specific immune surveillance and may in part contribute to the inefficiency of the metastatic process.

Additionally, several epithelial cell adhesion molecules, whose expression is extensively modulated during EMT, have been identified as potential activating/inhibitory ligands for NK cells. For example, ECAD is a known inhibitory ligand for NK cells (Schwartzkopff et al., 2007) and down regulation of its expression is a hallmark of EMT. Therefore, it is tempting to speculate that the modulation of ECAD expression could be another potential mechanism by which cells undergoing EMT may become more susceptible to NK-mediated cytotoxicity. Similarly, among other cell adhesion molecules, CADM1, which is identified as an activating NK ligand (Arase et al., 2005; Boles et al., 2005; Faraji et al., 2012), is frequently downregulated in multiple different malignancies and the inhibitory nectin protein receptor CD96 is implicated in promoting spontaneous metastasis (Blake et al., 2016b). Like NKG2DL, it would be important to assess whether these non-classical ligands and their receptors are also modulated during EMT.

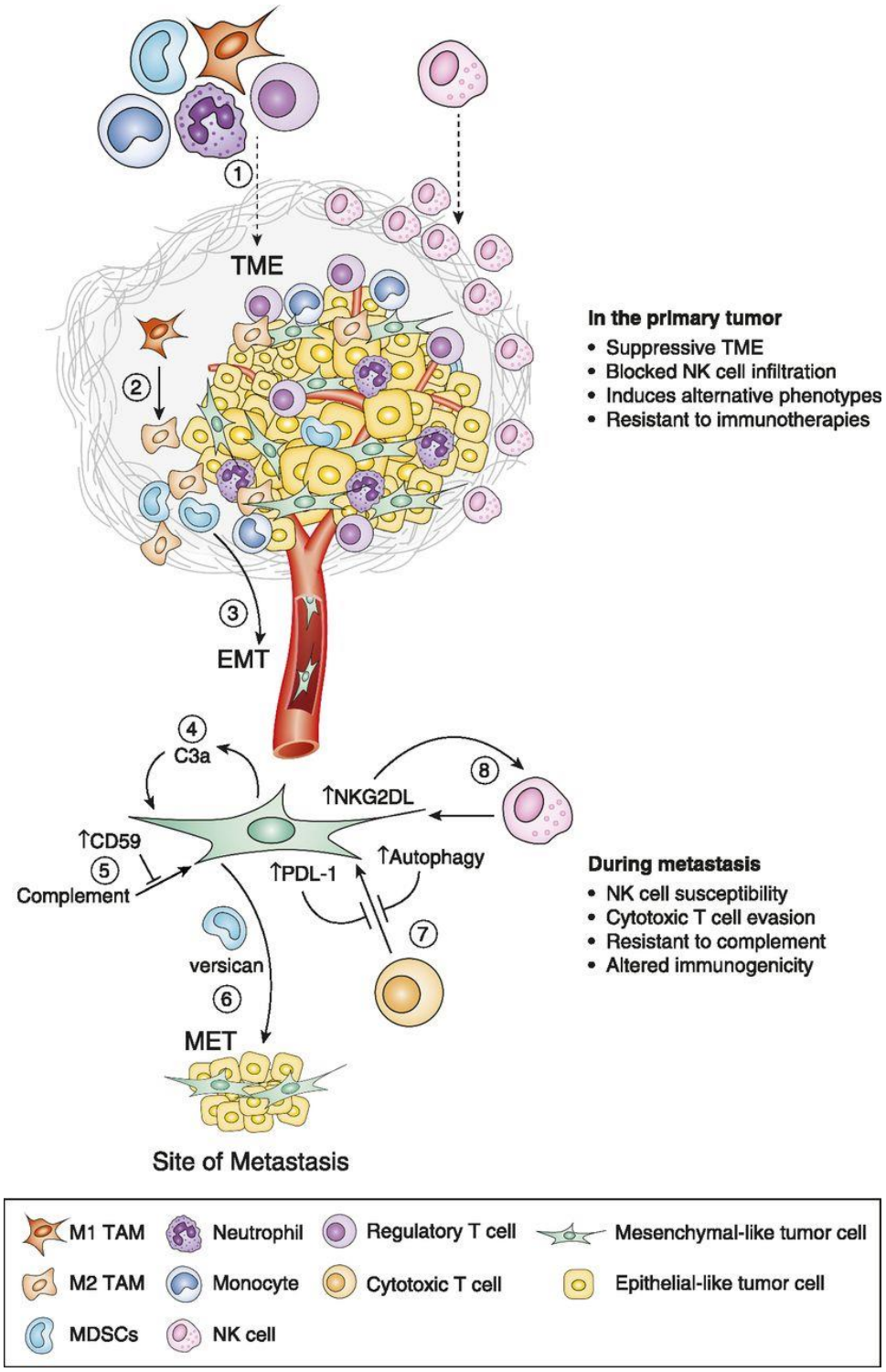

Figure 1-5 Cancer and immune cell interactions during tumor progression

(1) Differential recruitment of various immune cell types is modulated by the tumor microenvironment (TME). (2) Tumor-associated macrophages (TAMs), neutrophils, and Monocytes are converted to an anti-inflammatory phenotype. (3) M2 TAMs in conjunction with

Myeloid-derived suppressor cells (MDSCs) help drive EMT in the TME. These recruited cells also help suppress tumor-infiltrating lymphocytes. (4) Upon successful EMT, cells can maintain this phenotype via autocrine complement protein C3a production and (5) blocks complement-mediated cytotoxicity by upregulating CD59. (6) MDSCs can help metastasis formation by secreting versican to help the reverse process of mesenchymal to epithelial transition (MET) and seed the pre-metastatic niche at the distant site from the primary tumor. Increased PDL-1 and tumor autophagy aids in blocking cytotoxic T cell (CTL) (7) synapse formation and cytotoxicity. (8) This metastatic process can be limited by NK cell-mediated cytotoxicity by targeting upregulated NKG2D ligands.

Table 1 Summary of Cancer-Immune Cell Interactions and their Functional Consequences

Cell Type	In Primary Tumor	During Transition	At Metastatic site
Effects of Tumor Infiltrating Immune Cells on Cancer Cell EMT			
TAMs	• Recruited by CCL2[10,11] • Secretes: TGFβ, PDGF, EGF, TNFα, IL-1, and IL-6[5,10,14] • Induces EMT[5,10] • Promotes angiogenesis and tumor growth[5,9]	• Prevents anoikis[17,18] • Tumor cell survival[16,17,18] • Degrades ECM[16] • Aids intra- and extravasation[15]	• Promotes metastasis[10]
Monocytes	• Secretes: TGFβ, PDGF, EGF, TNFα, IL-1, and IL-6[5,10,14] • Induces EMT?	• Aids intra- and extravasation[20]	• pMOs inhibit metastasis[21]
Neutrophils	• Recruited by CXCL15 and HMGB1[25] • Promote tumor growth?	• NETs trap cancer cells[23,24]	• Promotes metastasis[23,24]
MDSCs	• Secrete: MMPs, CXCL5, CXCL12, VEGF, bFGF, HGF, TGFβ[36] • Induces EMT • Promote tumor growth[32,33,34,35]		• Establish premetastatic niche by versican secretion[38]
Platelets		• Shields from NK cells[30] • Sustains EMT via TGFβ[28] • Aids in extravasation[29]	• Promotes metastasis[26]
Complement	• Induces EMT via C3a → TWIST[40] • Promotes Tumor Growth[40]	• C3a sustains EMT[40]	
T cells	• Promotes tumor growth[42] • Induces EMT via Tregs?		
Impact of EMT on Immune cell Functions			
Macrophages	• Promotes M1→M2 via IL-4, GM-CSF, TGFβ[21] • M2 via TGFβ induced IRAK-M[41] • Promotes tumor growth[41]		
Complement		• Induction of CD59 on Tumor cells[51] • Resistance to CDC[51]	• Promotes metastasis[51]
T cells	• Induction of Tregs[42] • Evasion of CTL[43]	• Tumor Autophagy blocks CTL synapse formation[43]	• Promotes metastasis[42]
NK cells	• Secretion of soluble ligands[61] • Promotes tumor growth?	• Induces activating ligands (NKG2DL[61], Nectins[63,64]) • Suppression of inhibitory ligands (Ecad)[61] • Secreted soluble NKG2DL leads to suppression?	• Inhibits metastasis[60]

Chapter 2 EMT Leads to NK-Mediated Immune Surveillance in Lung Cancer

EMT Confers Susceptibility to NK-Mediated Cytotoxicity

Modeling EMT In Vitro

There are numerous ways to induce EMT in epithelial-like cancer cells as discussed previously. The multifunctional cytokine transforming growth factor-β (TGFβ) is a potent inducer of EMT and promotes tumor progression in late stage tumors (Zavadil and Bottinger, 2005). We have selected a cytokine-induced method whereby the addition of exogenous TGFβ leads to the autocrine production of endogenous TGFβ and maintains the signaling leading to fulminant EMT. Expression of TGFβ is frequently up-regulated in cancers, including NSCLC (Kim et al., 1999), and is correlated with enhanced invasion, metastasis, and poor prognosis for patients with lung cancer (Kong et al., 1999). In NSCLC, we have shown that TGFβ-induced EMT confers a migratory and invasive phenotype promoting metastasis (Keshamouni et al., 2009; Keshamouni et al., 2006), inhibition of EMT blocked tumor metastasis (Reka et al., 2011; Reka et al., 2010), and an EMT-associated secretory phenotype was predictive of outcomes in NSCLC patients (Reka et al., 2014), illustrating the biological and clinical significance of EMT in lung tumor progression.

Here we add 5ng/ml of TGFβ to human A549 lung adenocarcinoma cells and observe the changes over 72 hours. To model the reverse MET process, simply washing off the cell growth media and replacing with fresh media depletes the TGFβ pool and halts the cyclical TGFβ

signaling. Over the next 168 hours, cells will begin to reform cell-cell contacts and tight junction proteins will again be expressed (**Figure 2-1**). We believe this model mimics the process of an epithelial-like primary tumor that disseminates mesenchymal-like metastatic cells and the reversal of that phenotype to form overt metastases at a distant organ. In addition to the physical characteristics of EMT we have also explored alterations at the genetic and protein levels.

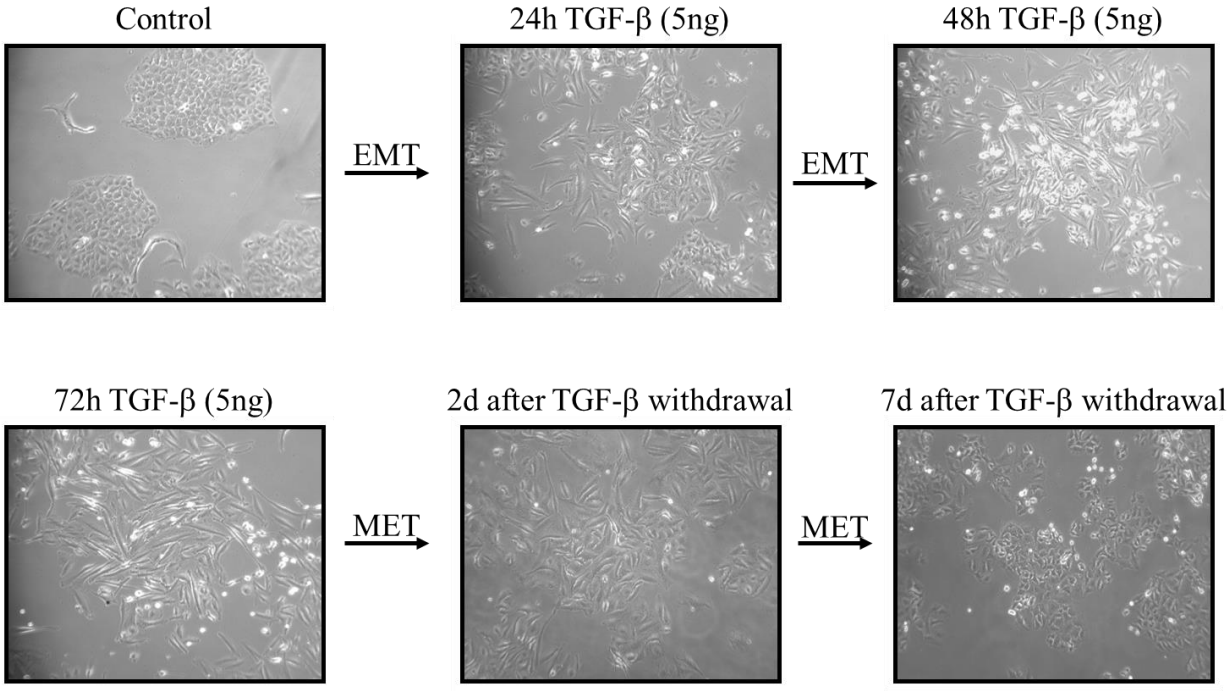

Figure 2-1 Morphological Phenotypes of A549 Cells Undergoing EMT and MET

A549 lung adenocarcinoma cells were serum starved for 24hrs then stimulated with 5ng/ml of TGFβ in 5% serum containing RPMI media. Images depict the morphological changes that occur during TGFβ-induced EMT. A549 cells complete EMT in 72hrs and upon washing off the extant media and replacing with fresh, untreated, complete media cells begin to undergo the reverse MET process. This is shown by the tight, cobblestone-like colonies that form and disperse and re-form.

EMT Differentially Regulates NK Ligands

We sought to assess what changes occur during EMT that could have an immunologically-relevant effect. Analysis of a previously obtained time course gene expression

profile during TGFβ-induced EMT (Keshamouni et al., 2009; Sartor et al., 2010), identified differential modulation of several NK activating ligands, in addition to the modulation of epithelial and mesenchymal markers (**Figure 2-2A**). We observed a significant time-dependent induction of mRNA for NK-activating ligands PVR, CADM1, ULBP2 and ULBP4 (**Figure 2-2B**). Similarly, among the known inhibitory ligands, ECAD expression was significantly suppressed but there was no significant change in the expression of MHCI in response to EMT. NK cell reactivity is regulated by the balance of activating and inhibitory receptor engagement by ligands expressed on the target cells.

Given the significant advances in T cell immunotherapies, we wanted to explore if any alterations occurred in regards to antigen presentation as well. Further, we assessed the gene expression profile for: MHCI and II for antigen presentation abilities; antigen processing associated proteins TAP1, TAP2; and Tapasin, costimulatory CD40, CD80, and CD86; and the checkpoint inhibitory ligand PDL1 **(Figure 2-2C**). We found no significant alterations in any of these traditionally T cell-related pathways. We further utilized a proteomic screen of the same A549 cells that have undergone EMT after 72 hours. Protein expression correlated with alterations in NK ligand mRNA expression (**Figure 2-2D**). Most strikingly there was a 23-fold increase of CADM1 after 72 hours.

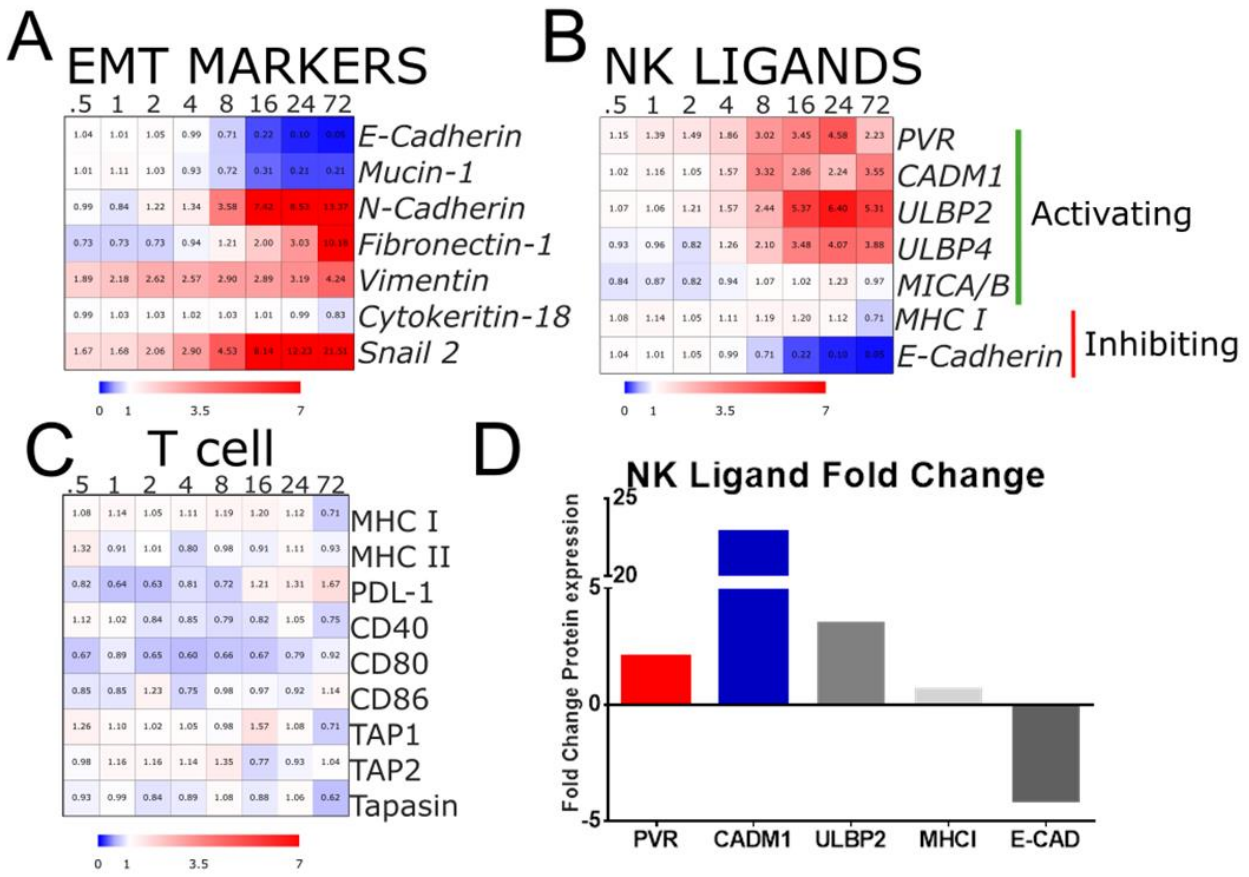

Figure 2-2 EMT Differentially Regulates NK Ligands but not T cell-Associated Molecules

(**A**) Heat map (blue: down regulation, red: up regulation) representing fold-changes, from 0hrs to 72hrs, time course of differentially expressed EMT markers and
(**B**) NK ligand or (**C**) T cell-associated genes during TGFβ-induced EMT, from previously published gene expression profile data set (GSE17708) (Sartor et al., 2010).
(**D**) Protein expression of NK Ligands from an unpublished proteomic screen.

EMT Confers Enhanced Susceptibility to NK Cytotoxicity

Collectively, the increased gene expression of activating ligands and decreased expression of inhibitory ligands in tumor cells undergoing EMT suggests a potential increased susceptibility to NK-mediated cytotoxicity. To test this, we assessed the susceptibility of a panel of human cancer cell lines to NK-mediated cytotoxicity before and after EMT. All cell lines were stimulated with TGFβ to induce EMT. EMT was validated morphologically and by loss of epithelial markers and gain of mesenchymal markers. These transitioned cells were then co-

cultured with the human NK cell line, NK92mi, to assess tumor cell-specific killing by flow cytometry (**Figure 2-3A**). NK92mi cells were selected to have a ready and consistent population of effector cells. Significant variance can occur based on the sources of effector NK cells especially with freshly isolated human NK cells. To further reduce the potential variance that may occur in using NK92 cells, we specifically selected NK92mi cells that constitutively express IL-2. Having constant and consistent IL-2 removes the issues that can arise with quality between sources, dosage times, and amounts.

Consistent with the ligand expression profile, we observed a significantly increased susceptibility to NK-mediated cytotoxicity in EMT cells compared to the controls at all tumor-to-NK cell ratios tested (**Figure 2-3B-F**). The EMT-induced susceptibility to NK cells was not specific to lung cancer cells, but was also observed in breast (**Figure 2-3E**) and colon (**Figure 2-3F**) cancer cells suggesting that this may be a more general phenomenon in response to EMT.

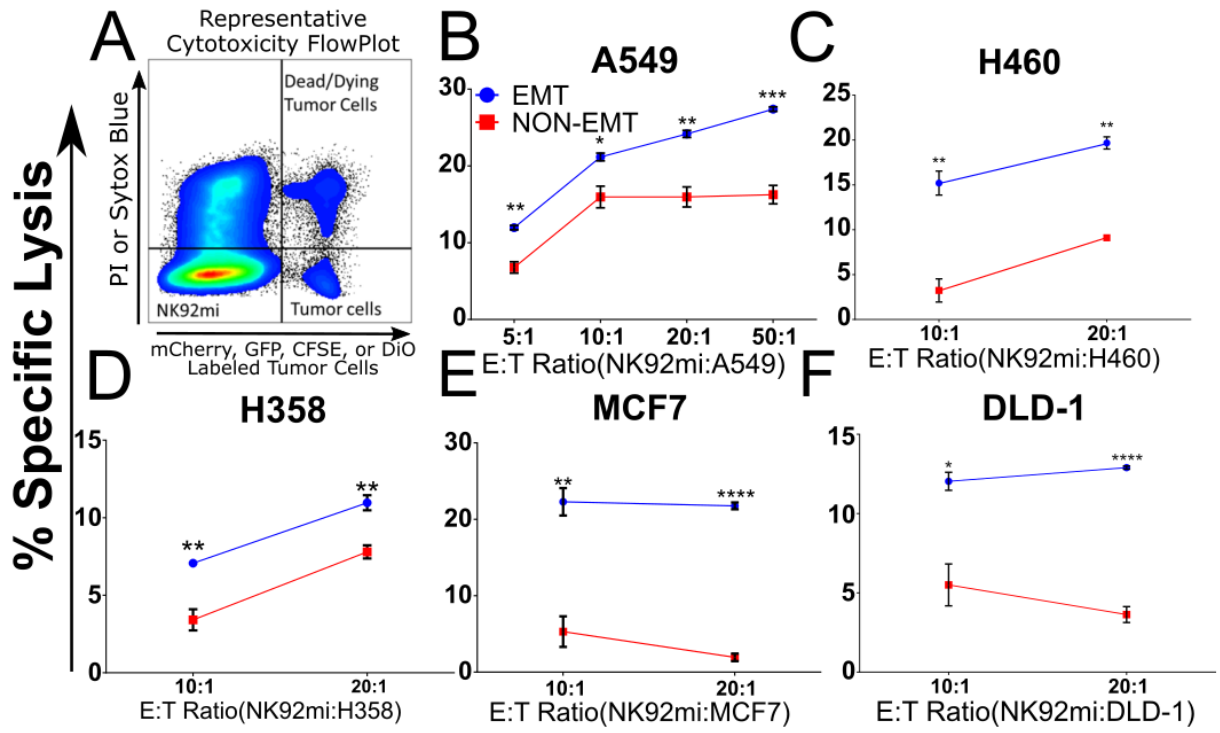

Figure 2-3 EMT Promotes Susceptibility to Human NK-Mediated Cytotoxicity

(**A**) Representative flow cytometric plot of cytotoxicity assay showing locations of effector, NK92mi, (fluorophore null cells) and target cells (fluorophore positive) and their exclusion DNA binding dye status (viability indicator).
(**B-F**) NK92mi mediated cytotoxicity plots after 4 hours of co-culture at indicated E:T ratios per cell type and treatment. Cell lines were treated with TGFβ (5ng/ml) for 3, 6, 12, 6, 6 days to induce optimum EMT, as assessed by complete ECAD down-regulation and induction of vimentin or N-cadherin, in A549 (**B**), H460 (**C**), H358 (**D**), MCF7 (**E**), and DLD1 (**F**), respectively. Data represents triplicate mean±SEM and two-tailed unpaired, t-tests were performed. All experiments were repeated at least twice. *P < 0.05, **P < 0.01, ***P < 0.001.

EMT Susceptibility Occurs with Unmodified Murine and Human NK Cells

Similarly, murine lung cancer cells also demonstrated increased susceptibility to NK-mediated cytotoxicity after EMT (**Figure 2-4**). In this case, CD45+, NK1.1+, CD3e- NK cells isolated from total splenic cells, and after overnight culture, were used as effector cells against murine lung cancer cells 344SQ as targets.

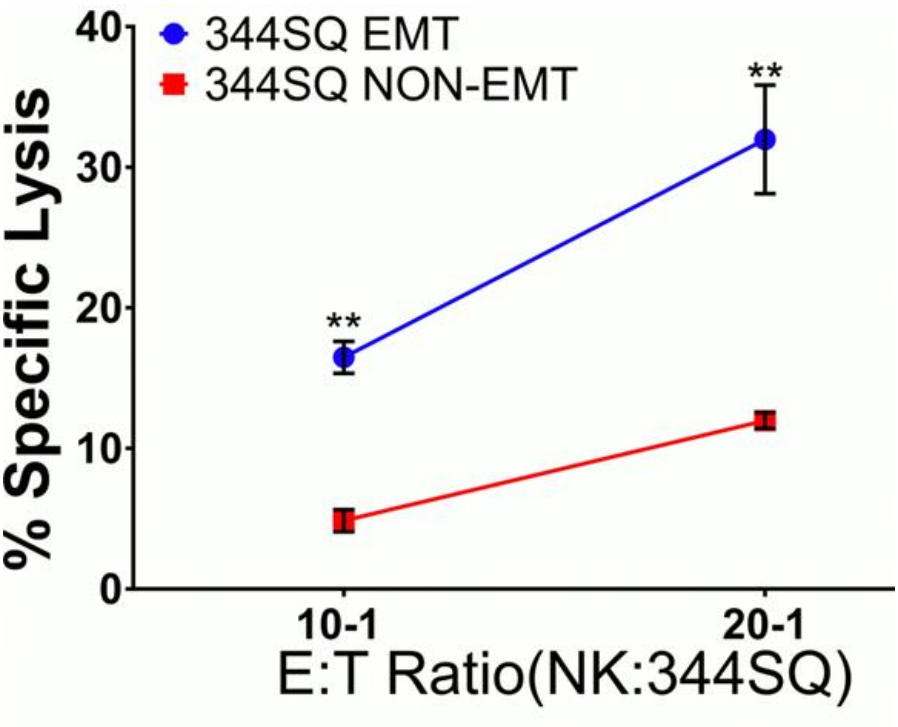

Figure 2-4 Murine NK cells Preferentially Target Cancer Cells That Had Undergone EMT

Isolated splenic murine NK cells were rested overnight and then co-cultured with TGFβ-induced EMT 344SQ murine epithelial lung cancer cell line. Data represents triplicate mean±SEM and two-tailed unpaired, t-tests were performed. All experiments were repeated at least twice. *P < 0.05, **P < 0.01, ***P < 0.001.

To demonstrate that this increased cytotoxicity is not specific to NK92mi cell line, human NK cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors as effectors against EMT and control A549 cells as targets. The differential susceptibility between EMT and control A549 cells was also observed when primary PBMC derived NK cells were used as effectors (**Figure 2-5**). K562 cells were used as positive controls to ensure cytotoxic capable NK cells were isolated. Taken together, this indicated that the differential killing of EMT cells was neither Nk92mi-dependent nor cell line-dependent.

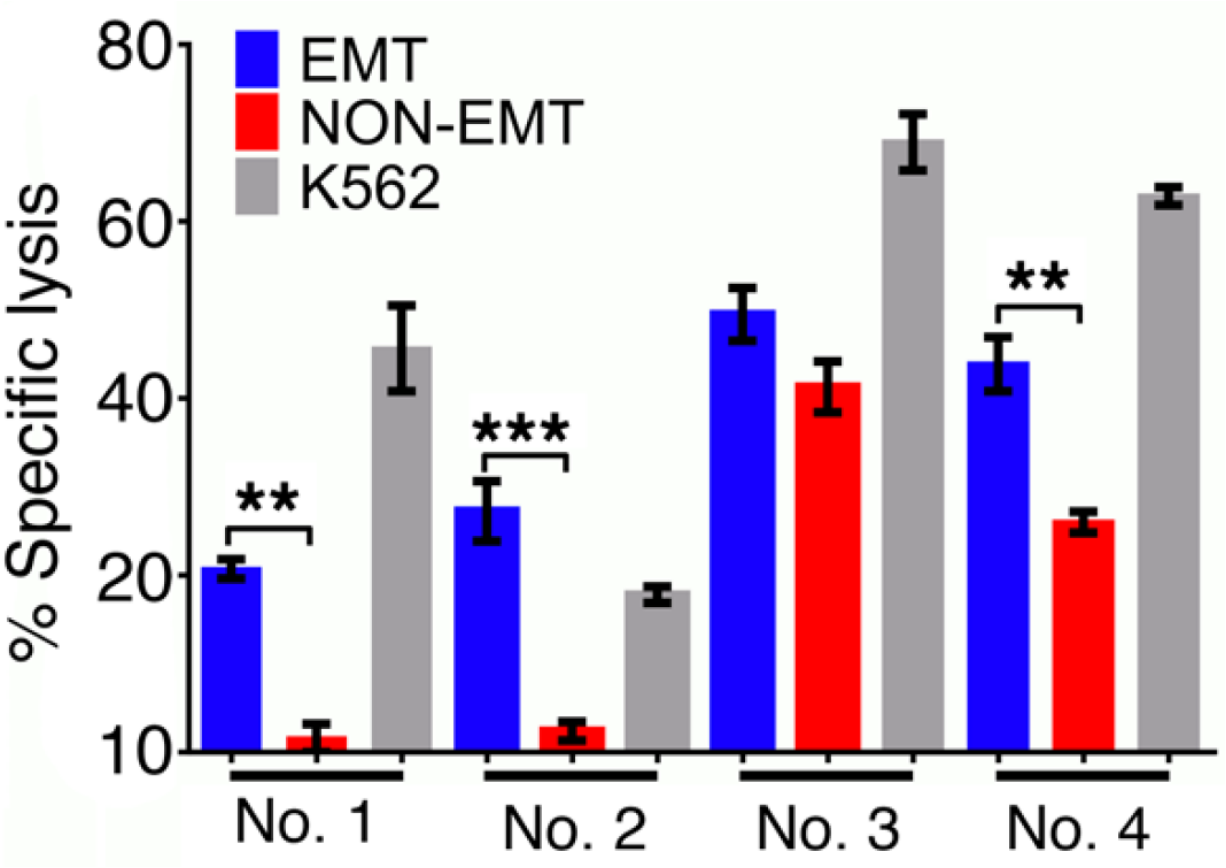

Figure 2-5 Isolated Human Peripheral Blood NK Cells Target Cells That Had Undergone EMT

Freshly isolated human peripheral blood-derived NK cells were used as effector cells (E:T, 10:1) against A549 cells. K562 cells were used as a positive control for cytotoxicity. Data represents mean±SEM and two-tailed, unpaired, t-tests were performed. *P < 0.05, **P < 0.01, ***P < 0.001.

NK Cells Require Direct Contact and Degranulate Upon Killing Target Cells

We next sought to elucidate the cytotoxic mechanism utilized by NK cells. First, we wanted to investigate whether target cell killing was mediated by direct contact or by secreted factors that trigger a response and subsequent killing. Here, we placed NK cells and target cells in a 0.3 micron transwell system. This pore size was selected to allow passage of soluble factors but not cells. Upper chambers contained NK cells or NK cells with either EMT or NON-EMT A549 cells. The lower chambers contained EMT or NON-EMT A549 target cells only. This was

to also allow direct contact to observe if factors were secreted that mediated the cytotoxicity observed in a co-culture system (**Figure 2-6A**). Secondly, we wanted to assess if the differential killing shown previously was due to impaired cell-cell conjugation. Here, we co-cultured labelled NK92mi cells with either labelled A549 EMT or NON-EMT cells at a 1:1 ratio and assessed for conjugation via flow cytometry. We observed an increasing frequency of relative conjugation over time. There was no difference in conjugation between groups over 1 hour of co-culture (**Figure 2-6B**).

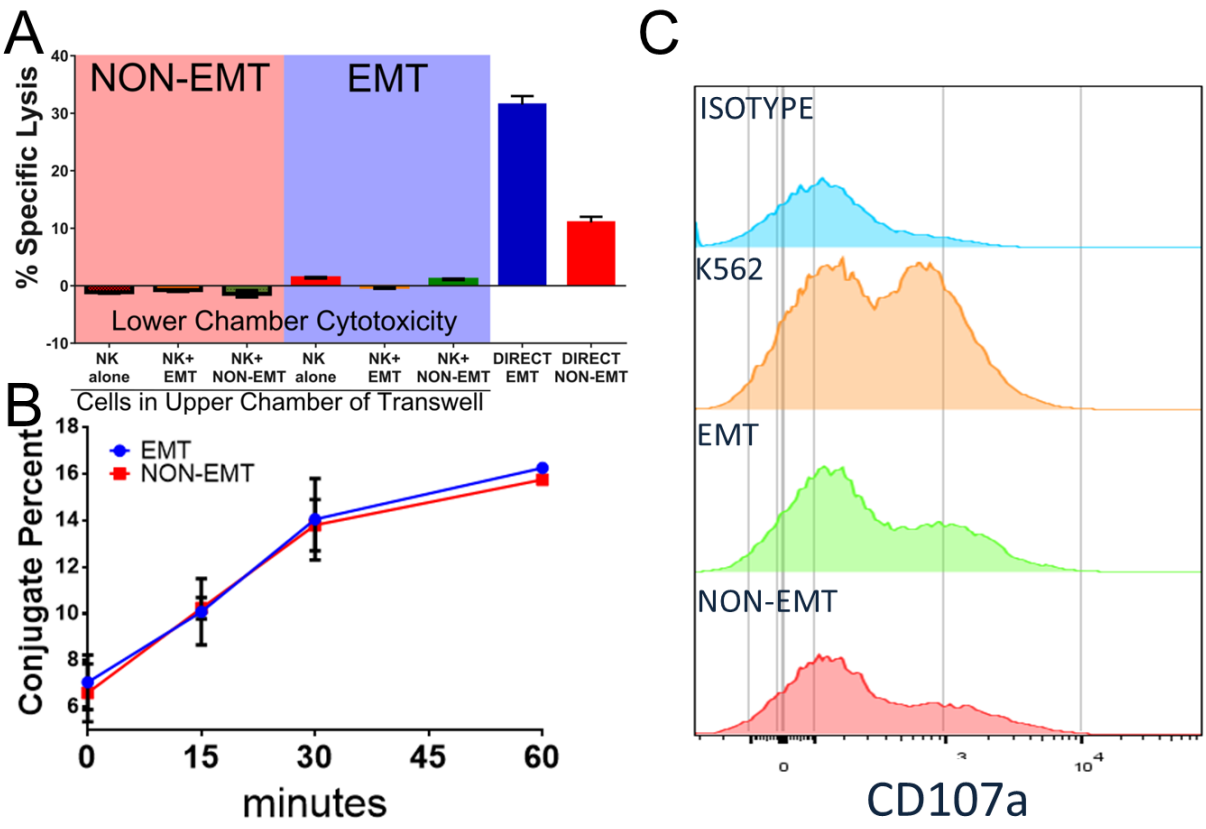

Figure 2-6 Cell-Cell Contact is Required for NK-Mediated Killing of A549 Cells

(**A**) NK cells were either placed in the upper chamber of a .3 micron transwell alone, with EMT A549 cells, or NON-EMT A549 cells. In the lower chamber target cells, EMT or NON-EMT, were placed and cytotoxicity was measured. As a control, directly contacting EMT and NON-EMT A549 cells with NK92mi cells cytotoxicity assays were performed.
(**B**) NK92mi cell conjugate with target cells equally and in a time-dependent manner.

(**C**) NK92mi cells degranulate, as determined by CD107A surface expression, upon contact with target cells. K562 cells were used as a positive control.

Finally, we sought to determine if the NK cells were degranulating during cytotoxicity. After 4 hours of NK92mi co-culture with various targets, with K562 again being used as a positive control, we observed that NK92mi cells degranulate as shown by CD107A staining (**Figure 2-6C**). CD107A, also known as lysosomal associate membrane protein 1, is not typically expressed on the surface of cells, but when lysosomes fuse to the outer plasma membranes during degranulation CD107A is exposed and can be used as a surrogate marker for degranulation. Taken together, our data suggests a contact-dependent recognition and a predominately perforin/granzyme lytic pathway used for target cell cytotoxicity. This is corroborated by the timing of our experiments as well, as 4 hours is likely too short for purely FAS/FASL-mediated cell death (Hassin et al., 2011; Itoh et al., 1991).

These experiments revealed an alteration of NK ligands in response to TGFβ-induced EMT and that EMT confers susceptibility to NK-mediated killing *in vitro*. This would suggest the potential of a metastasis-specific immune surveillance mechanism *in vivo*. To this end, we employed various murine models of metastasis with modulation of NK cells.

Chapter 3 NK Cells Control Metastasis

NK Cell Depletion Allows for Experimental Metastasis of EMT A549 Cells

In earlier studies we demonstrated that TGFβ-induced EMT in A549 cells triggers a migratory and invasive phenotype *in vitro* and promotes metastasis *in vivo* (Keshamouni et al., 2006; Reka et al., 2010). Inhibition of TGFβ signalling, which prevents EMT, blocked metastasis of A549 cells (Reka et al., 2010) indicating an EMT-dependent metastasis model.

NK Depletion Validation

Next, to demonstrate the direct link between EMT and NK-mediated immune surveillance *in vivo*, we induced EMT in A549-GFP cells as described earlier (Reka et al., 2010) and injected cells into the tail vein of two groups of $RAG1^{-/-}$ mice. $RAG1^{-/-}$ mice do not have T and B cells but have functional NK cells. Further, to confirm an NK-specific immune surveillance, NK cells were depleted with either an antibody against the activating receptor NK1.1 or Asialo-GM1 (ASGM1) (**Figure 3-1A-B**). Both antibodies can give lasting depletion and with subsequent doses can render the effective NK cell pool non-existent. To assess the extent of depletion, splenic cells were isolated and analysed by flow cytometry. We observed a nearly 90% reduction in the number of NK cells (CD45+/NK1.1+/CD3ε- cells) with ASGM1 treatment (**Figure 3-1B**).

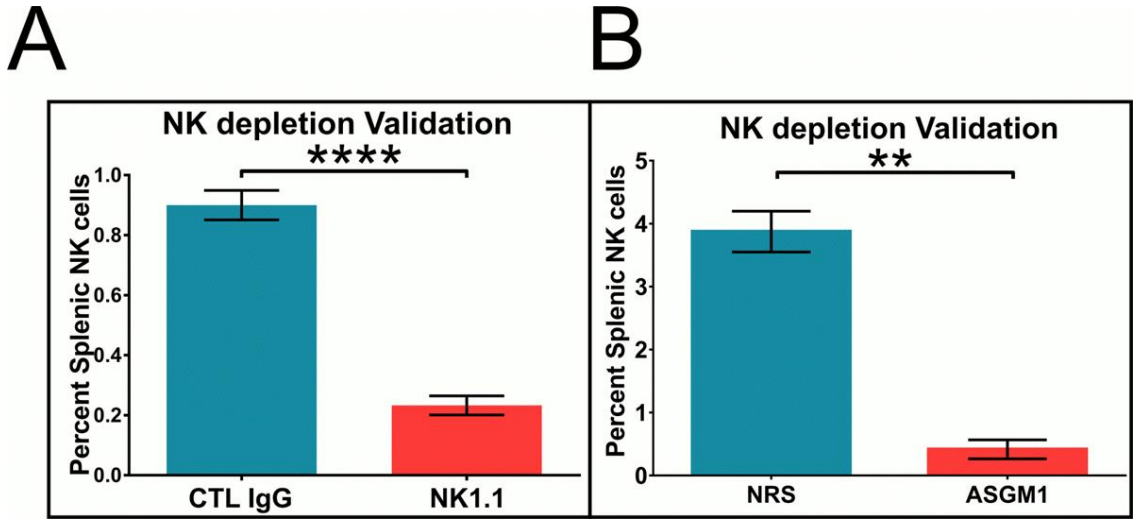

Figure 3-1 NK Depletion Validation

(**A**) A single injection of 200µg of anti-NK1.1(clone: PK136) or CTL IgG was administered to C57BL/6 mice i.p. and 5 days later spleens were harvested and assessed for NK cells (CD45+,NK1.1+, CD3e-), n=3 per group, mean±SEM is shown.
(**B**) A single injection of 25µl of anti-Asialo GM1(ASGM1) or control normal rabbit serum(NRS) was administered to C57BL/6 $RAG1^{-/-}$ mice and 7 days later spleens were harvested and assessed for NK cells (CD45+,NK1.1+, CD3e-), n=3 per group, mean±SEM is shown. Significance was determined by unpaired, two-tailed, t-test.

NK Cells Target EMT A549 Cancer Cells In Vivo

After depletion of NK cells and subsequent injection of EMT A549 cells, mice were sacrificed after 6-8 weeks to assess lung metastasis. We observed metastatic lung nodules only in the mice treated with ASGM1 and not in the control group (**Figure 3-2A**). Overt lung nodules were counted and a representative stereo dissection microscopic fluorescent image is shown with GFP positive lung nodules (**Figure 3-2B**). Further confirming the lack of lung nodules in control NRS treated mice, histological analysis was performed as well (**Figure 3-2C**). Strikingly, the control mice with NK cells rarely showed micro-metastases, highlighting the importance of NK cell surveillance. This experiment demonstrated EMT-induced susceptibility to NK-mediated immune surveillance, either in circulation or in the lungs, *in vivo*.

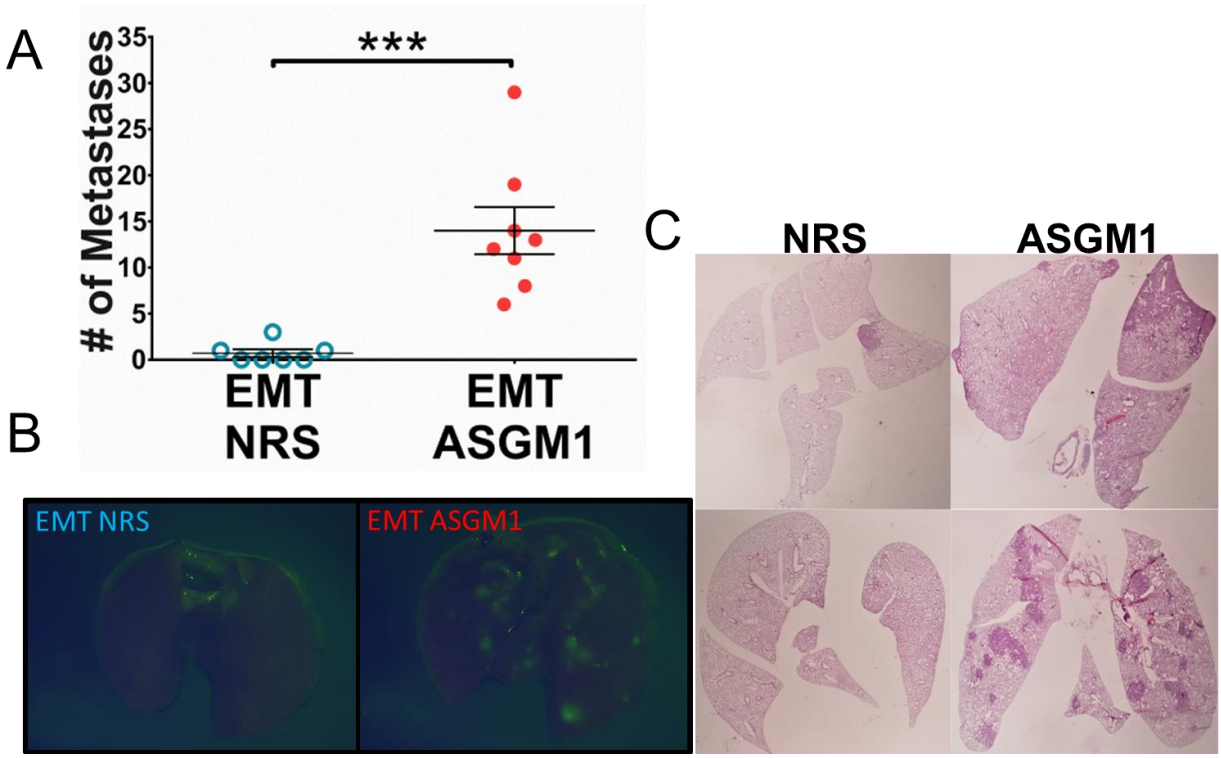

Figure 3-2 NK cells Control Experimental Metastasis of EMT A549 Cells

(**A**)To assess experimental metastasis A549 cells were treated with TGFβ (5ng/ml) *in vitro* and injected through tail vein into RAG1$^{-/-}$ mice. After 8 weeks lung were harvested to assess tumor burden via overt lung nodule counts. Data shown represents two independent experiment, n=3-4 for each group, and pooled results shown. Error bars are SEM and Mann-Whitney U test was performed, *P < 0.05, **P < 0.01, ***P < 0.001.
(**B**) Fluorescent images of example lungs from NRS and ASGM1-treated mice.
(**C**) H&E staining of two mice from each group of NRS or ASGM1 treatments showing the disparate degree of lung nodules present.

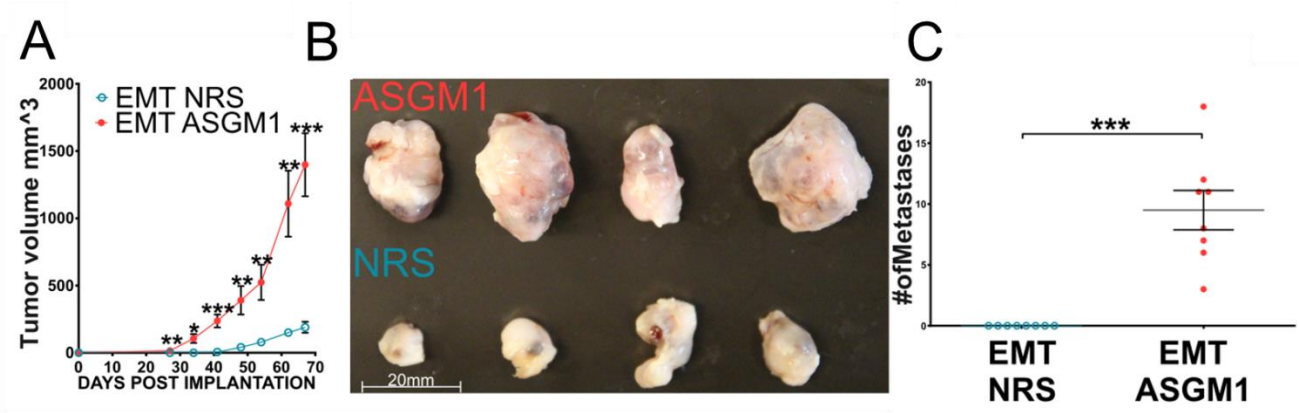

Figure 3-3 NK cells Target EMT A549 Cells at Primary Tumor Site

(**A**) To assess the location of where NK cells target EMT A549 cells, transitioned A549 cells were implanted into the dorsal flank of RAG1$^{-/-}$ mice with weekly NK cell depletion by ASGM1. Error bars are SEM and Student's unpaired, two-tailed t-test was performed, *P < 0.05, **P < 0.01, ***P < 0.001.
(**B**) Tumors were excised and scale bar is 20mm.
(**C**) Spontaneous metastatic burden was assessed by over lung nodule counts. Data shown represents two independent experiment, n=3-4 for each group, and pooled results shown. Error bars are SEM and Mann-Whitney U test was performed, *P < 0.05, **P < 0.01, ***P < 0.001.

To further determine the location at which NK cells may target susceptible EMT A549 cells, we performed the same experiment as described above, but instead of a tail vein injection we did a subcutaneous dorsal flank implantation. Here we observed a robust growth inhibition of tumor mass in the mice with NK cells present (**Figure 3-3A-B**). Furthermore, we observed metastatic nodules in the lungs of the mice with depleted NK cells (**Figure 3-3C**). Given that these cells were pre-treated with TGFβ and have already undergone EMT, we still needed to explore a more physiologically applicable model.

NK Cell Deletion Allows Spontaneous Metastasis without Effecting Primary Tumor Growth

To assess the role of NK cells in a spontaneous metastatic model, we implanted 10^6 untreated A549 cells in two groups of RAG1$^{-/-}$ mice on either side of the dorsal flank. These cells were EDTA lifted to retain as much of the protein expression on the surface as possible. One

group of mice were treated weekly with ASGM1 antibody to deplete NK cells. Interestingly, depletion of NK cells had no effect on the primary tumor growth (**Figure 3-4A**). In contrast, we observed spontaneous lung metastasis only in the mice that are depleted of NK cells **(Figure 3-4B**), suggesting the presence of an NK-mediated metastasis-specific immune surveillance. Together with the observations in (**Figure 3-2**), this also suggests an EMT-dependent mechanism.

To assess the role of NK cells in metastasis in an immunocompetent host, we employed a syngeneic murine model of Lewis lung carcinoma (LLC) cells. We subcutaneously implanted LLC cells in C57BL/6 hosts with and without NK cell depletion using ASGM1 antibody. LLC cells grow very aggressively in the syngeneic host, but do not metastasize. Similar to the xenograft model above, depletion of NK cells had no effect on the primary tumor growth (**Figure 3-4C**), but we observed spontaneous lung metastasis only in mice that were treated with ASGM1 antibody **(Figure 3-4D**). In a parallel experiment, we used NK1.1 antibody instead of ASGM1 to deplete NK cells and observed similar results (**Figure 3-5A-B**). Since there is no single method that can exclusively deplete only NK cells, our data with ASGM1 (which is also known to effect basophil populations) and NK1.1 Ab (which is also known to deplete NK-T cells) showing similar effects supporting an NK cell-dependent phenomena.

The potential contribution of T and B cells to the observed metastasis-specific immune surveillance was assessed by implanting LLC cells in T and B cell-deficient $RAG1^{-/-}$ mice. Notably, we observed similar spontaneous metastasis only upon NK depletion (**Figure 3-4F**), and there was no effect on the growth of the primary tumor with and without NK cell depletion (**Figure 3-4E**). We also observed similar results when 344SQ cells were implanted in C57BL/6 mice with and without ASGM1 NK depletion (**Figure 3-5C-D**). Collectively, similar

observations in five different models above demonstrate the presence of a NK cell-dependent, metastasis-specific immune surveillance mechanism. While, EMT was implicated in various experimental results, we did not explore the specific signaling that needs to occur to induce EMT or metastasis. Since our previous *in vitro* data was based on TGFβ-induced EMT we sought to explore the relevance of that signaling *in vivo*.

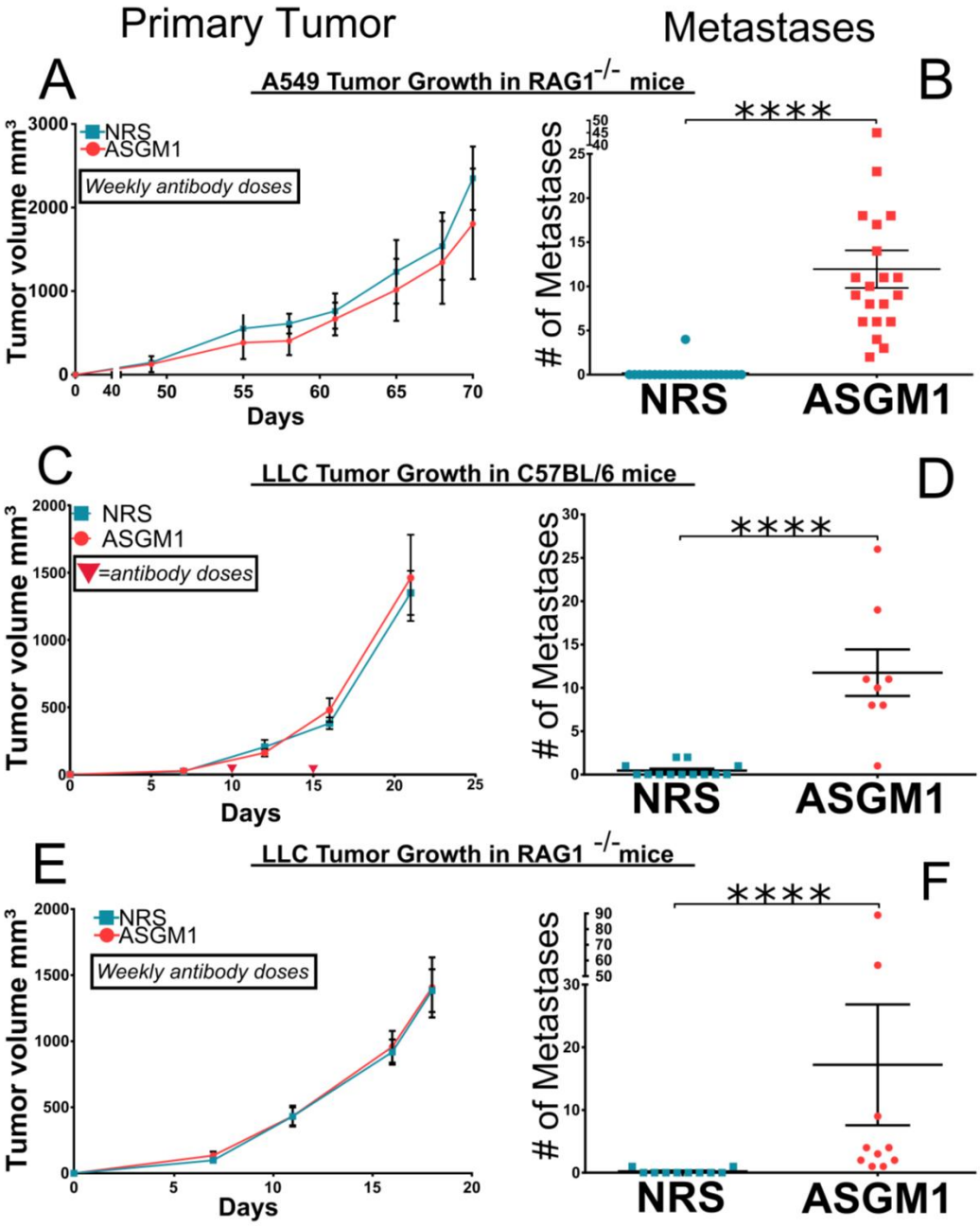

Figure 3-4 NK Cell Depletion Allows Spontaneous Metastatic Spread without Effecting Primary Tumor Growth

(**A**-**F**) To assess the effect of NK cell depletion on primary tumor growth and metastasis indicated cell lines were implanted subcutaneously under the dorsal flanks of RAG1$^{-/-}$ or C57BL/6 mice. Mice were treated weekly with anti-Asialo GM1 antibody (ASGM1) to deplete NK cells or normal rabbit serum (NRS) as control.
(**A**, **C**, and **E**) Primary tumor growth was monitored and mean-tumor volumes are plotted with errors bars as SEM. Representative data from a single experiment of at least duplicates.

(**B**, **D**, and **F**) Overt lung nodules were counted on the excised lungs to assess spontaneous metastasis. Mouse strains and tumor cell implants are designated. Error bars are SEM and Mann-Whitney U test was performed, *P < 0.05, **P < 0.01, ***P < 0.001. Data represents at least two experiments, n=4-5 per group, and pooled data is shown.

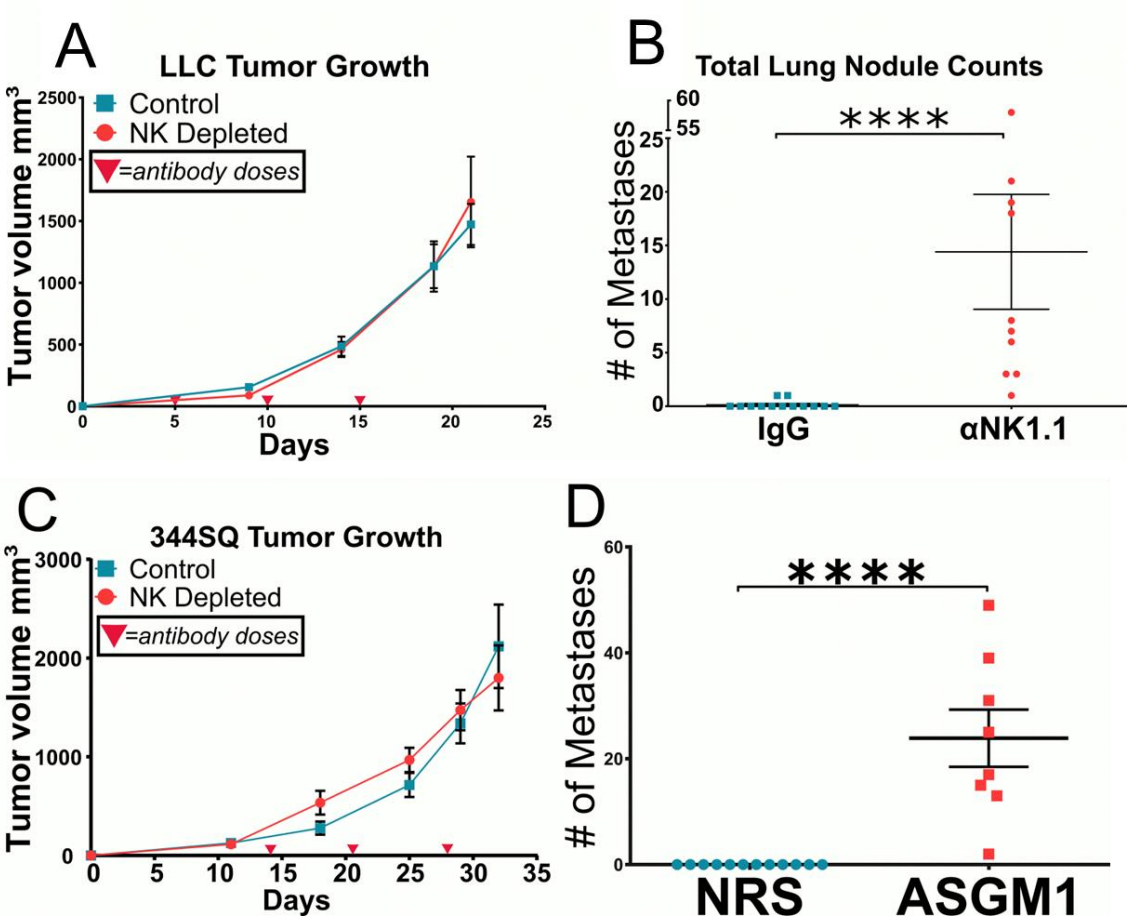

Figure 3-5 Additional Spontaneous Metastatic Murine Models of Lung Cancer

(**A**) To assess the effect of NK cell depletion on primary tumor growth and metastasis of murine LLC tumor cells, cells were implanted subcutaneously into the dorsal flanks of in C57BL/6 mice. 200μg Anti-NK1.1 was administered every 5 days to deplete NK cells. Mean±SEM shown.
(**B**) Spontaneous metastatic lung nodules were quantified from (**A**), pooled data from two experiments is shown, n=5 mice per group. Error bars are SEM and Mann-Whitney U test was performed, *P < 0.05, **P < 0.01, ***P < 0.001.
(**C**) To assess the effect of NK cell depletion on primary tumor growth and metastasis of murine 344SQ tumor cells, cells were implanted subcutaneously into the dorsal flanks of in C57BL/6 mice. Mice were treated with ASGM1 at indicated time points to deplete NK cells. Mean±SEM shown.
(**D**) Overt lung nodules were counted on the excised lungs to assess spontaneous metastasis. Pooled data is shown from two experiments n=4 mice per group. Error bars are SEM and Mann-Whitney U test was performed, *P < 0.05, **P < 0.01, ***P < 0.001.

TGFβRI Signaling Is Critical for TGFβ-Induced EMT In Vitro and Metastasis In Vivo

To assess the requirement for TGFβ signaling to induce EMT and initiate metastasis, we deleted TGFβRI from A549 cells. We did not delete the TGFβ production from A549 cells since murine TGFβ could, putatively, be used by the human A549 cells to induce EMT and metastasis. We utilized a CRISPR/Cas9-mediated approach to delete the TGFβRI and then subsequently assessed for the ability to undergo EMT via TGFβ treatment. To this end, we have a stable Cas9 expressing A549 cell line and purchased pro-viral plasmids that encode the various guide RNAs, an mCherry fluorophore, and a puromycin resistance gene. A549 cells would then be infected with the lentiviruses made from the plasmids and using both puromycin and mCherry positivity we attained a 100% resistant and mCherry positive cell population.

As shown in (**Figure 3-6**) we found that guide RNA construct "A" failed to block EMT, construct "B" effectively halted EMT and "C" partially blocked EMT. A549 EMT is characterized both morphologically and, more critically, by ECAD downregulation and mesenchymal marker induction shown here by vimentin. Clonal selection was not performed, but positive selection by both puromycin resistance and mCherry fluorescence was utilized to ensure that cells were selected and transfected fully. We did not do clonal selection to retain as much heterogeneity in the original cell population as possible. We elected to use construct "B" cell line for our subsequent *in vivo* studies given the full blockade of EMT (**Figure 3-6**).

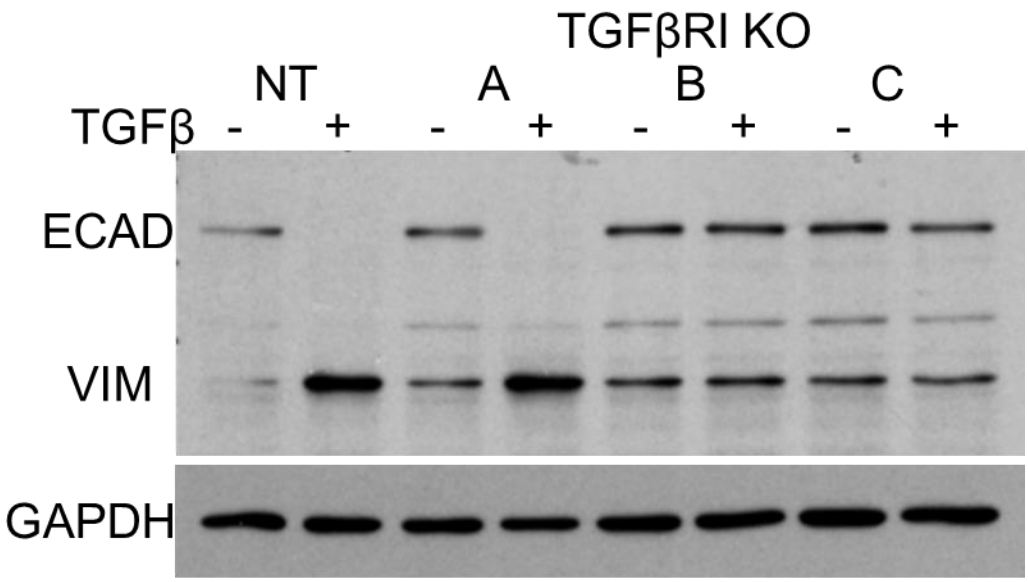

Figure 3-6 TGFβRI KO A549 EMT

Western immunoblot analysis of TGFβRI KO A549 cell clones treated with and without 5ng/ml TGFβ and allowed to transition for 72 hours. ECAD = E-Cadherin, VIM = Vimentin and GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase.

Utilizing the TGFβ insensitive A549 clone "B" we wanted to ensure that TGFβ-induced EMT was critical for A549 metastasis *in vivo*. To this end, we implanted TGFβRI KO A549 cells into the dorsal flanks of mice and depleted NK cells weekly with ASGM1. We again observed no primary tumor growth variations with or without NK cells (**Figure 3-7A**). Contrary to our previous results using TGFβ responsive A549 cells, when NK cells are depleted we did not find a significant increase in metastasis from control A549 cells with NK cells present (**Figure 3-7B**). This has also been shown in other models of metastatic breast cancer, whereby canonical TGFβ signaling was inhibited (Tang et al., 2017). The few metastases in these mice could be due to a small population of cells in which TGFβRI was not deleted fully, an inherent trade off of not clonally selecting cell lines.

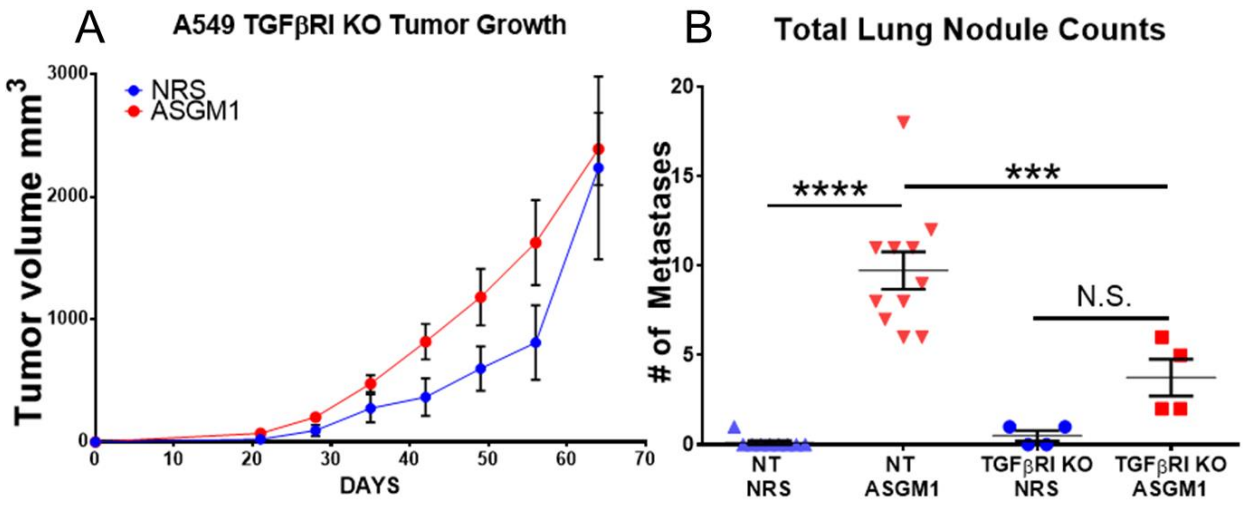

Figure 3-7 TGFβRI is Necessary for A549 Metastasis

(**A**) To assess the role that TGFβ and TGFβRI plays primary tumor growth of A549, cells were implanted in the dorsal flanks of RAG1$^{-/-}$ mice. NK cells were depleted with weekly administration of ASGM1
(**B**) Spontaneous metastatic burden was assessed from the groups in (**A**) Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001.

The previous sets of *in vitro* and *in vivo* experiments have confirmed that EMT is critical for metastasis in our model and that NK cells are critical for metastatic control. The depletion strategies utilized with ASGM1 and NK1.1 and the murine models of fully immune competent and RAG1$^{-/-}$ mice lends credence to the notion that it is conventional NK cells rather than any other cell types affected by these antibodies. The intersection of depleted cell types in these indicates that NK cells are responsible for metastatic control. Going forward with this data, we sought to determine the molecular mechanisms of how NK cells specifically target these transitioning cells to halt metastatic spread.

Chapter 4 Molecular Mechanism of Enhanced NK Cytotoxicity to EMT Cells

Loss of ECAD Expression Sensitizes Tumor Cells to NK Cytotoxicity Via KLRG1

Classically, inhibitory signals on NK cells are mediated by receptors that recognize MHCI molecules. However, recent studies demonstrate that NK cells also express other receptors such as KLRG1 that can recognize MHC-independent inhibitory signals including ECAD or other adhesion molecules (Schwartzkopff et al., 2007). Many adhesion proteins are susceptible to enzymatic cleavage, and exploiting this fact can aid in narrowing possible NK ligands that are mediating differential killing of EMT cells. To test if there was a trypsin-labile protein responsible for either activating or inhibiting NK cell cytotoxicity of EMT A549 cells, we induced EMT with TGFβ in culture, as described previously, and lifted to the cells with EDTA then subsequently treated the A549 cells with .25% trypsin for 0, 5, or 10 minutes (**Figure 4-1**). Here, there is only an increase in NK-mediated cytotoxicity when NON-EMT cells were treated with trypsin, indicating removal of an inhibitory ligand or ligands. However, the subsequent cytotoxicity was not fully restored to EMT levels. Furthermore, there was no increase or change in the cytotoxicity observed in the EMT group, indicating that there likely is not a trypsin-labile protein responsible for activating NK cells.

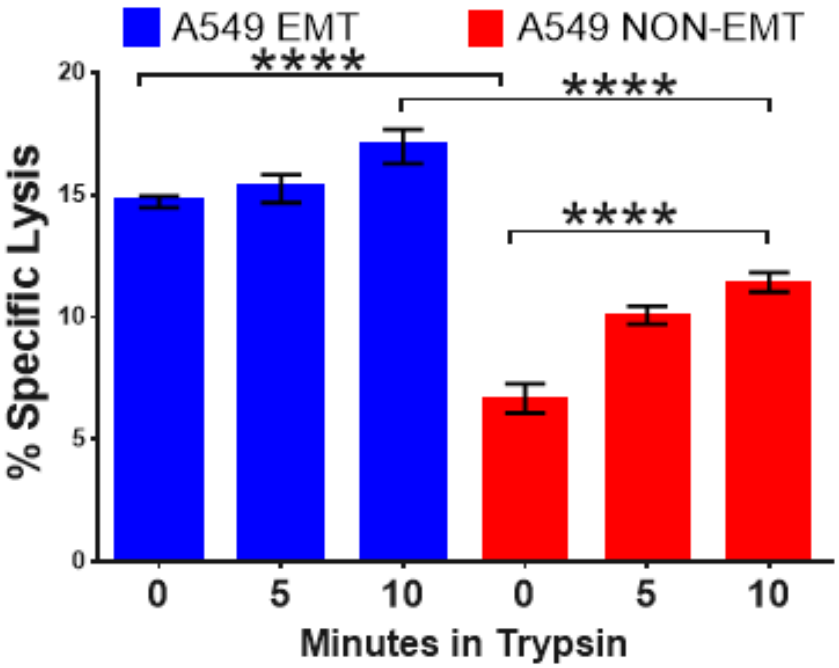

Figure 4-1 A Trypsin Labile Protein Inhibits NK Cytotoxicity in A549 Cells

To assess if there was an activating or inhibiting ligand that was trypsin-labile, A549 cells were induced with TGFβ to undergo EMT or not. These cells were then treated with trypsin for 0, 5, or 10 minutes. These cells were then subjected to co-culture with NK92mi cells and cytotoxicity was measured by flow cytometry. Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001.

ECAD/KLRG1 Axis Inhibits NK Cell Cytotoxicity

Since ECAD, as a hallmark of EMT, is down-regulated with a concomitant increase in the susceptibility to NK cell-mediated cytotoxicity, we reasoned that ECAD may be an important inhibitory signal that protects epithelial cells from NK cell-mediated cytotoxicity. Further, ECAD is known to be trypsin-labile. Data from previous experiments indicated an inhibitory signal is originating from a trypsin-labile protein. To specifically test ECAD, we inhibited ECAD expression using three different siRNA molecules in A549 (**Figure 4-2A**), then assessed their susceptibility to NK cell cytotoxicity using NK92mi cells as described previously. We observed that ECAD inhibition increased susceptibility of A549 cells to NK-mediated

cytotoxicity (**Figure 4-2B**). However, the magnitude of cytotoxicity did not reach the levels observed in A549 cells after EMT. Next, we determined whether ECAD-induced inhibitory signaling is indeed mediated through KLRG1 on NK cells. siRNA-mediated inhibition of KLRG1 expression in NK92mi cells, as measured by flow cytometry (**Figure 4-2C**) enhanced their cytotoxicity against the NON-EMT control as well as EMT-induced tumor cells (**Figure 4-2D**) demonstrating that KLRG1 mediates ECAD-induced inhibitory signaling. Moreover, this inhibition is rather potent as even the greatly reduced levels of ECAD on EMT cells still played an inhibitory role on NK cells. Inhibiting the ECAD/KLRG1 axis did not fully recapitulate the enhanced cytotoxicity against A549 cells undergoing EMT, suggesting that it may also require the induction of an activating ligand.

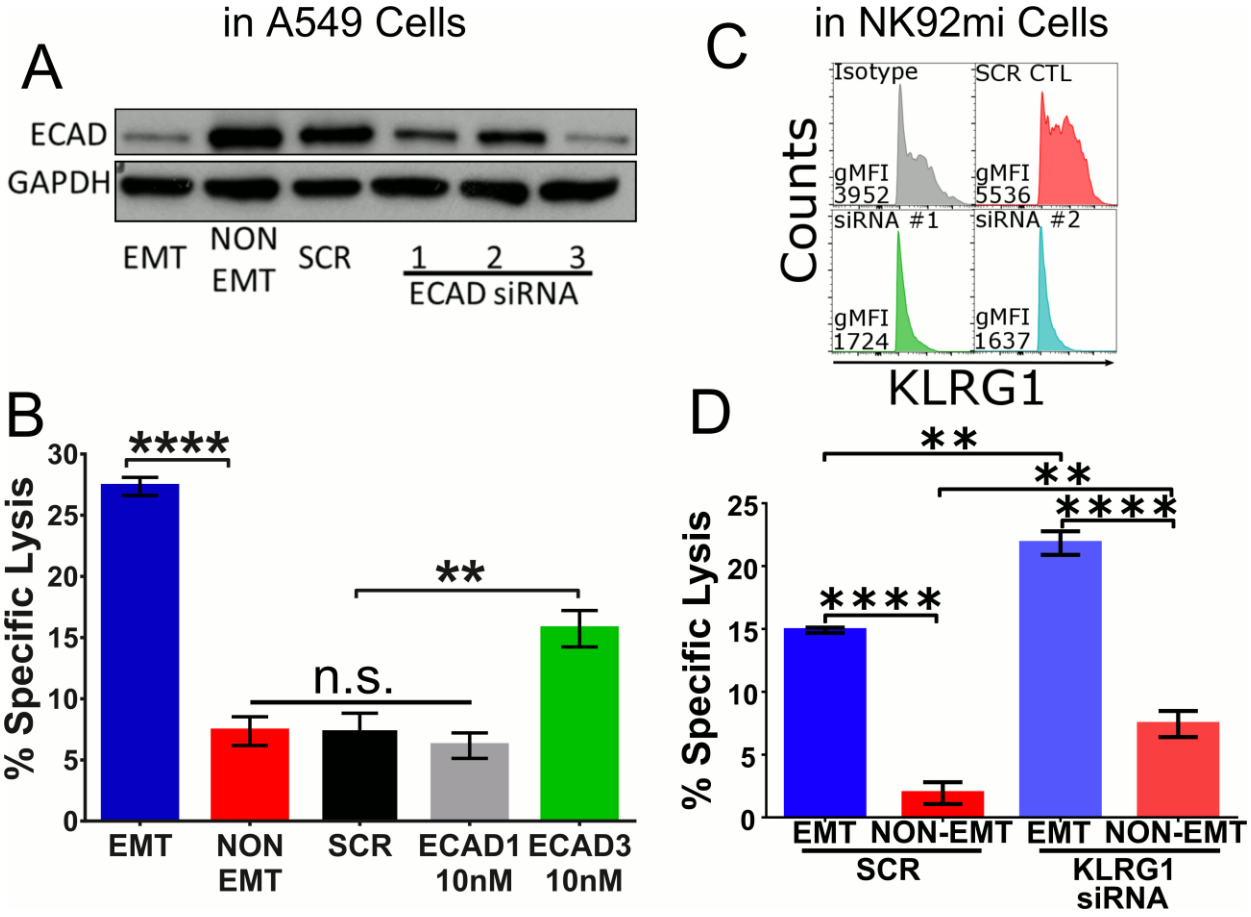

Figure 4-2 Loss of ECAD Expression Sensitizes Tumor Cells to NK-Mediated Cytotoxicity through KLRG1

(**A**) A549 cells were transfected with 10nM of scrambled (SCR) or 3 different ECAD-specific siRNA molecules. After 24hrs, cells were treated with (EMT) or without (NON-EMT) TGF-β (5ng/ml) for 72hrs. ECAD and GAPDH expressions were assessed by western immunoblotting.
(**B**) Susceptibility to NK cytotoxicity was assessed using NK92mi cells as effectors, as described for Figure 1. Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001.
(**C**) NK92mi cells were transfected with 10 nm of SCR or KLRG1-specific siRNA. After 72hrs, KLRG1 expression was assessed by flow cytometry.
(**D**) NK92mi cells from (**C**) were used as effectors against EMT or non-EMT A549 cells in the NK cytotoxicity assay. Mean±SEM is shown and two-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001. All experiments were repeated twice and data shown is representative of one experiment.

NKG2D Receptor is Not Involved in the EMT-induced Susceptibility to NK-Mediated Cytotoxicity

Since we observed a robust induction of NKG2D ligands ULBP2 and ULBP4 during EMT, we tested the potential involvement of NKG2D as an activating NK cell receptor mediating tumor cell killing after EMT. We assessed the involvement of NKG2D by independently blocking its expression, using siRNA, and functionality, using neutralizing antibodies, in NK92mi cells. K562 cells, whose NK-mediated cytotoxicity is partly NKG2D dependent, were used as a positive control (Li et al., 2008). Interestingly, inhibition of expression or function of NKG2D receptors had no effect on A549 tumor cell killing before or after EMT (**Figure 4-3A-B**). This line of evidence is consistent in fact that murine NKG2D receptors do not engage human NKG2D ligands; given that previous experiments have shown that murine NK cells kill human A549 cells *in vivo*, this suggests that alternative activating receptors may be critical for NK cell recognition of A549 cells post-EMT and are conserved across mouse and human genomes.

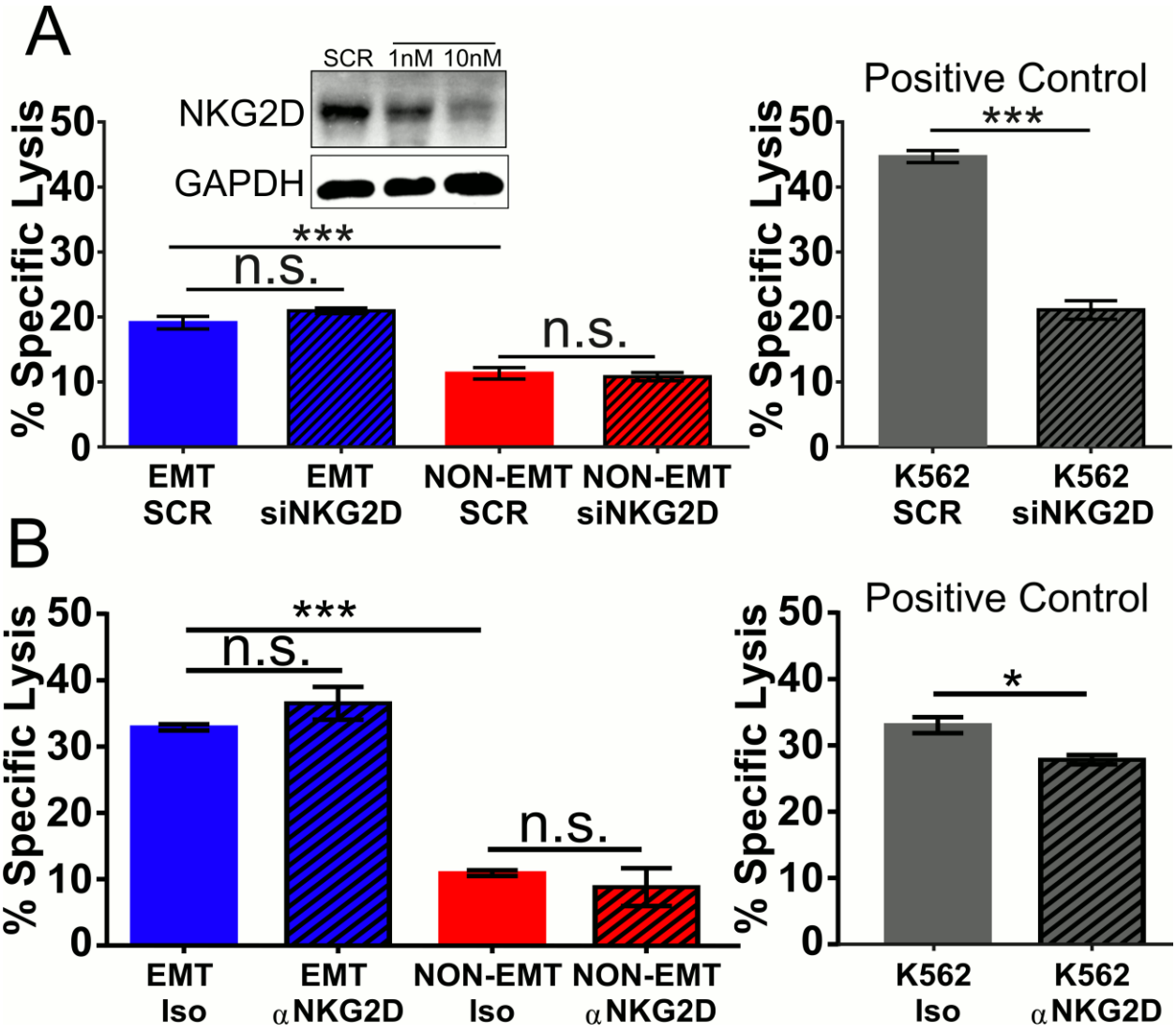

Figure 4-3 NKG2D Receptor is Not Involved in EMT-Induced Susceptibility to NK-Mediated Cytotoxicity

(**A**) NK92mi cells were transfected with 10 nm of scrambled (SCR) or NKG2D-specific siRNA. After 72 hours, NKG2D expression was assessed by western immunoblotting (inset), and these cells were used as effectors against A549 cells that are treated with (EMT) or without (Non-EMT) TGF-B (5ng/ml) for 72 hrs, in NK cytotoxicity assay.
(**B**) NKG2D receptors were neutralized by treating NK92mi cells with anti-NKG2D receptor antibody (50 ug/ml) 45 minutes prior to co-culturing them with EMT or non-EMT A549 cells in the NK cytotoxicity assay. All experiments were repeated twice representative data of one experiment shown. Mean±SEM shown and two-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001. For Positive Control K562 cytotoxicity, Mean±SEM is shown and two-tailed, unpaired, t-tests were performed *P < 0.05, **P < 0.01, ***P < 0.001.

CADM1 Expression Mediates Tumor Cell Susceptibility to NK Cytotoxicity

CADM1 Deletion Reduces NK Cell Cytotoxicity

Given that neutralizing NKG2D receptors had no effect, we tested the role of CADM1, the next most abundant NK ligand induced during EMT, as a potential activating ligand mediating EMT-induced susceptibility to NK cells. CADM1 is identified as a tumor suppressor in lung cancer and its expression is lost in up to 40% of patients with NSCLC (Kikuchi et al., 2006; Kuramochi et al., 2001; Murakami, 2002). Interestingly, CADM1 has also been shown to form heterophillic interactions with an immunoglobulin family receptor known as Class I MHC-Restricted T Cell-Associated Molecule (CRTAM) that serves as its cognate receptor and expressed on activated NK cells, suggesting a role for CADM1 in immune surveillance (Boles et al., 2005).

To determine the role of CADM1 in NK-mediated cytotoxicity, we temporally deleted CADM via siRNA knockdown (**Figure 4-4A**). These CADM1 deficient cells underwent TGFβ-induced EMT equally as well as scramble control cells and had no effect on proliferation (**Figure APP 3**). Further, upon co-culture with NK92mi cells, cytotoxicity was significantly reduced in both EMT and NON-EMT cells (**Figure 4-4B**). This result led us to check another method of CADM1 inhibition by permanent deletion in A549 cells. We developed stable A549 cells with CADM1 knockout by genome-editing using CRISPR-Cas9 technology. Inherent to our Cas9 system, when cells are transfected they also become mCherry positive. We created (A549-CADM1 KO) together with corresponding non-targeting control cell line (A549-NT) (**Figure 4-4C**) and assessed the susceptibility, before and after EMT, to NK cell cytotoxicity. We observed that CADM1 inhibition abrogated EMT-induced susceptibility of A549 cells to

NK92mi cytotoxicity (**Figure 4-4D**). Strikingly, the general reduction of the primary donor-derived NK cell cytotoxicity (**Figure 4-4E**) indicates a more globally applicable method of evading NK cells and not just specific to NK92mi cells. Together with ECAD data (**Figure 4-2**), this demonstrates that modulation of both ECAD and CADM1 expression can regulate tumor cell susceptibility to NK-mediated cytotoxicity *in vitro*.

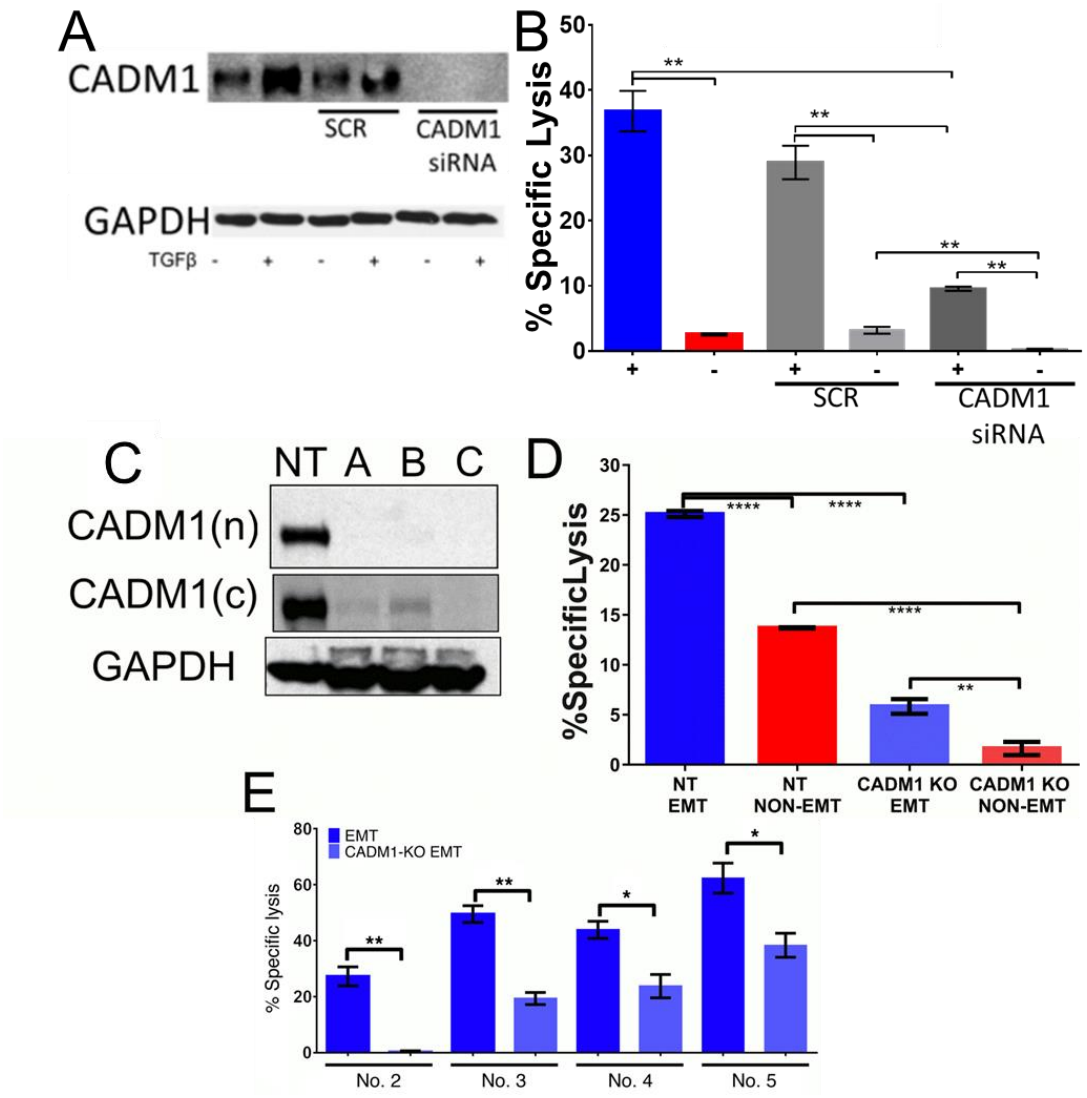

Figure 4-4 CADM1 Deletion Abrogates NK Cytotoxicity

(**A**) A549 cells were transfected with 10nM of scrambled (SCR) or CADM1-specific siRNA molecules. After 24hrs, cells were treated with (EMT) or without (NON-EMT) TGF-β (5ng/ml) for 72hrs. CADM1 and GAPDH expressions were assessed by Western immunoblotting.

(**B**) Susceptibility to NK cytotoxicity was assessed using NK92mi cells as effectors, as described for Figure 1. Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001.
(**C**) To stably knock-out CADM1, Cas9-expressing A549 cells were transduced with lentiviruses expressing three different CADM1 specific CRISPR sgRNA and a non-targeting (NT) control sgRNA. CADM1 knockout was assessed by western immunoblotting using two different CADM1 antibodies raised against C-terminal (CADM1-c) and N-terminal (CADM1-n) portions.
(**D-E**) Susceptibility of CADM1-KO A549 cells to NK cytotoxicity was assessed by co-culturing them with either NK92mi (D) cells or with primary human blood derived NK cells (E) from four different donors. In panel D, Mean±SEM shown and two-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001. In panel E, EMT controls are from Figure 1H as these experiments were performed simultaneously. Mean±SEM shown and two-tailed, unpaired, t-tests were performed *P < 0.05, **P < 0.01, ***P < 0.001.

CADM1 Deletion Is Not Required for NK-Target Conjugation

CADM1 has been implicated in activating NK cells via CRTAM (Arase et al., 2005; Boles et al., 2005). It is possible that CADM1 is also critical for immune synapse formation, given that it is an adhesion molecule. To explore this possibility, a conjugation assay was performed with CADM1 KO cells. If CADM1 was required for forming conjugations between cells it would suggest that CADM1 is not the activation ligand. Upon NK92mi addition to A549 target cells we found no alterations in the ability to form conjugates, as measured by flow cytometry (**Figure 4-5**). Tubes were vortexed prior to running to disrupt and reduce non-specific clumping of cells to ensure more accurate reading of true conjugates. This data indicates that CADM1 is truly activating NK cells rather than aiding in cytotoxic signaling of an alternate ligand or synapse formation.

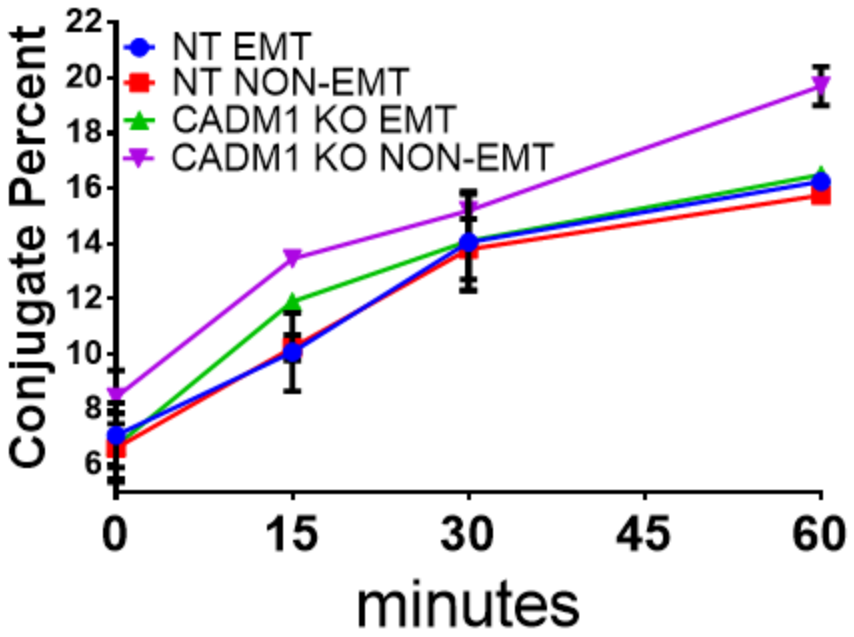

Figure 4-5 CADM1 Deletion Does Not Affect NK-Target Cell Conjugation in A549

NK92mi cells were allowed to conjugate with target cells at a 1:1 ratio for 0, 15, 30, and 60 minutes.

Chapter 5 CADM1 Expression Dictates NK-Mediated Immune Surveillance

Inhibition of CADM1 in Tumor Cells Enables Immune Evasion and Allows Metastasis

Since CADM1 is critical for signaling *in vitro* cytotoxicity to NK cells and was shown to abrogate the differential killing of EMT cells; we sought to determine if CADM1 mediated the NK surveillance of metastasizing cells *in vivo*. To assess the impact of CADM1 inhibition on tumor metastasis, we implanted NON-EMT A549-CADM1 KO and A549-NT cells into the dorsal flanks of $RAG1^{-/-}$ mice and assessed primary tumor growth and lung metastasis, as described previously. Notably, there was no difference in the kinetics of primary tumor growth between A549-CADM1 KO and A549-NT (**Figure 5-1A**). As expected, the tumors from the control A549-NT cells did not metastasize. However, there was a striking increase in overt lung metastasis from A549-CADM1 KO cells even without NK cell depletion, as assessed by gross counting (**Figure 5-1B**) and visualizing mCherry-positive tumor cell colonies in the lung (**Figure 5-1C**). This data indicates that *in vivo* CADM1 is a critical factor that leads to NK-mediated immune surveillance.

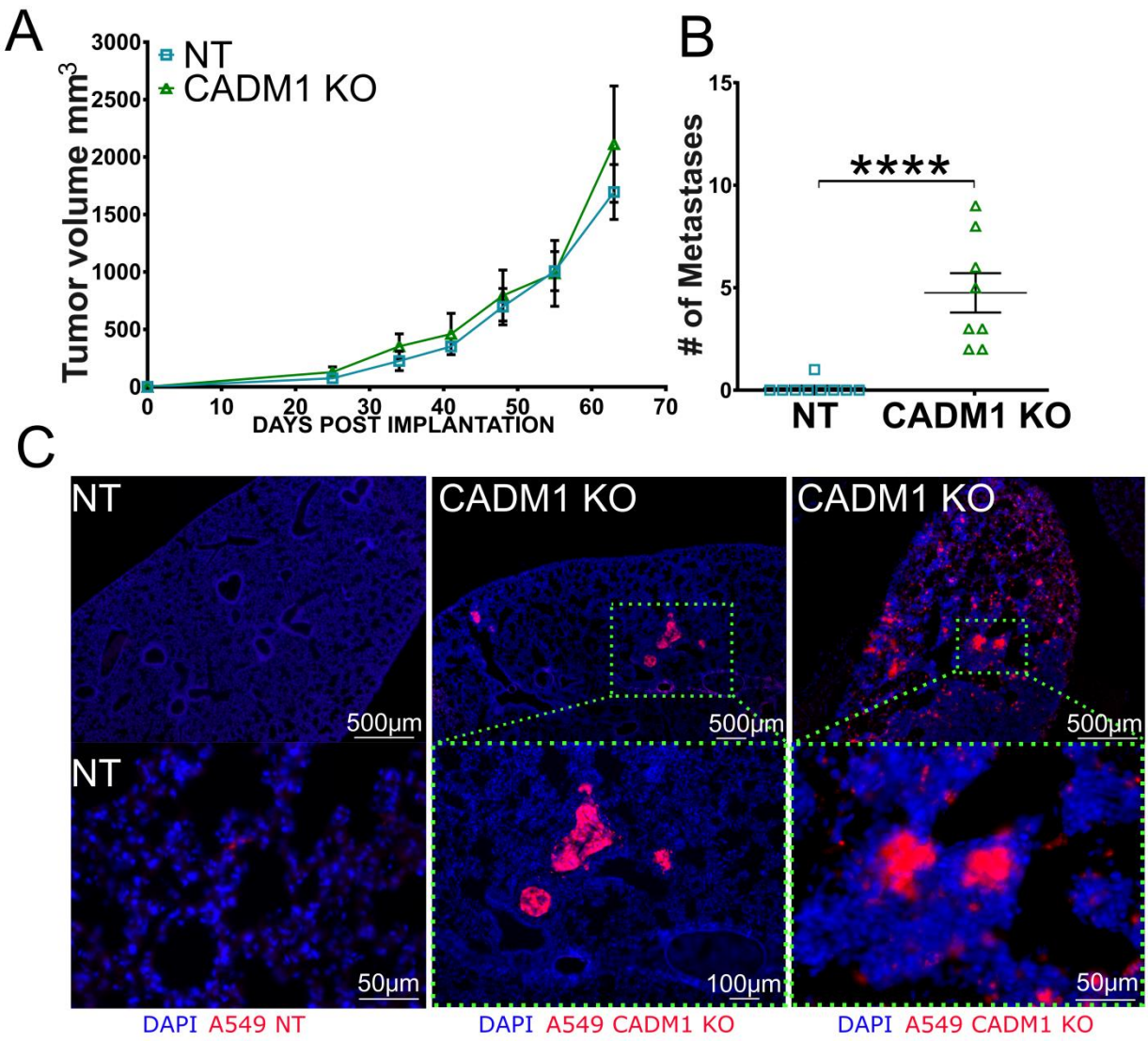

Figure 5-1 Inhibition of CADM1 in Tumor Cells Allows Spontaneous Metastasis without Effecting Primary Tumor Growth

(**A**) mCherry expressing NON-EMT CADM1 KO and control A549 cells were subcutaneously implanted into the dorsal flanks of RAG1$^{-/-}$ mice. Primary tumor growth was monitored and mean-tumor volumes are plotted, mean±SEM shown. Data is representative of one experiment (n=4-5 mice per group).
(**B**) Overt lung nodules were counted on the excised lungs to assess spontaneous metastasis. Data represents two independent experiments and pooled data is shown, error bars are SEM and Mann-Whitney U test was performed *P < 0.05, **P < 0.01, ***P < 0.001.
(**C**) Presence or lack thereof, of metastatic spread was further confirmed by visualizing mCherry positive tumor cells in the cross-sections of the lungs by immunofluorescence. Top row scale bars are all 500µM and lower row is 50µM, 100µM, and 50µM, respectively.

CADM1 Deletion Enhances Circulating Tumor Cell Numbers

Additionally, we investigated the role of CADM1 in circulating tumor cells. A549 mCherry cells were isolated from blood via cardiac puncture. After RBC lysis samples were subjected to an anti-EGFR, anti-EPCAM, and ant-CD133 antibody coated chip for cell capture and microscopic analysis. Here we show that whether NK cells are present or not, CADM1 sufficient A549 cells are present at the same levels (**Figure 5-2**). Consistent with our hypothesis, CADM1 depleted A549 cells were higher in number in the blood. This experiment points toward an NK surveillance mechanism that is mediated at the distant site, in this case the lungs. Taken together, our data indicates CADM1 inhibition alone is sufficient to allow metastasis which is otherwise blocked by NK cell immune surveillance. Putatively, loss of CADM1 observed in various cancers may be an immune evasive strategy employed by tumors.

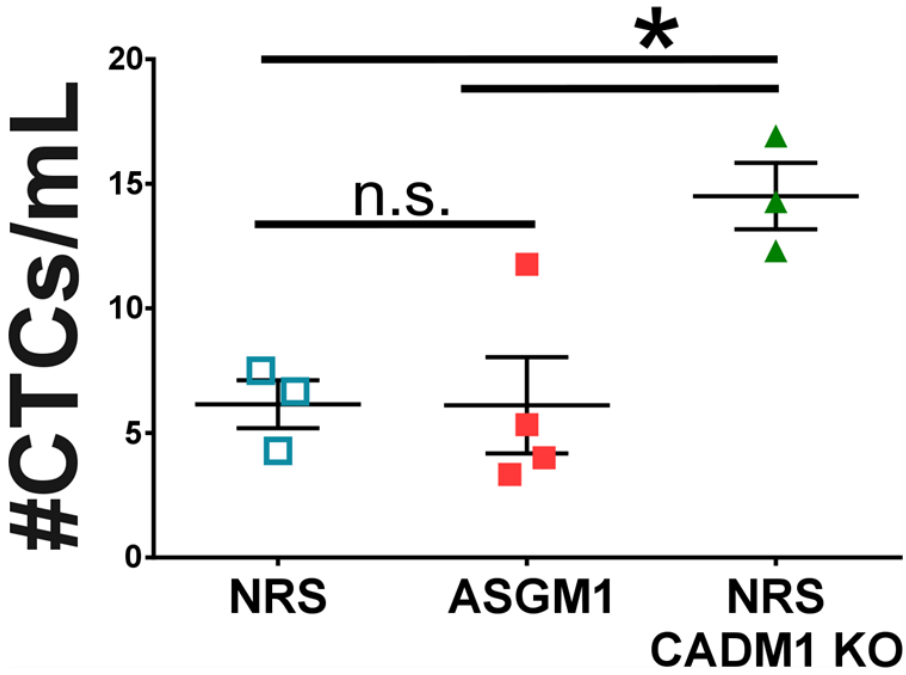

Figure 5-2 CADM1 Loss Increases Circulating Tumor Cells

(**A**) Peripheral blood of RAG1$^{-/-}$ mice was isolated upon tumor limits being reached. Red blood cells were lysed and whole blood was subjected to antibody coated chip capture. The circulating tumor cells were identified based on their mCherry positivity. Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001.

Restoring CADM1 Expression in Tumor Cells is Sufficient to Confer Susceptibility to NK Cytotoxicity

CADM1 Overexpression Does Not Effect Cell Growth In vitro

It is well documented that CADM1 expression is frequently lost in 40% of lung cancers either due to promoter hypermethylation, or loss of heterozygosity (LOH) (Murakami, 2005). Here we tested the efficacy of two approaches of restoring CADM1 expression on NK-mediated cytotoxicity. First, in a cell line with LOH for CADM1 (A549), we generated a stable cell line expressing a doxycycline (dox)-inducible CADM1 (A549 CADM1 OE). This was a double viral vector system, whereby one virus encoded a tetracycline-activated protein to bind response elements in a promotor on another expression vector with CADM1. Dox-induced overexpression of CADM1 had no effect on the growth and on TGFβ-induced EMT in A549 CADM1 OE cells (**Figure 5-3**).

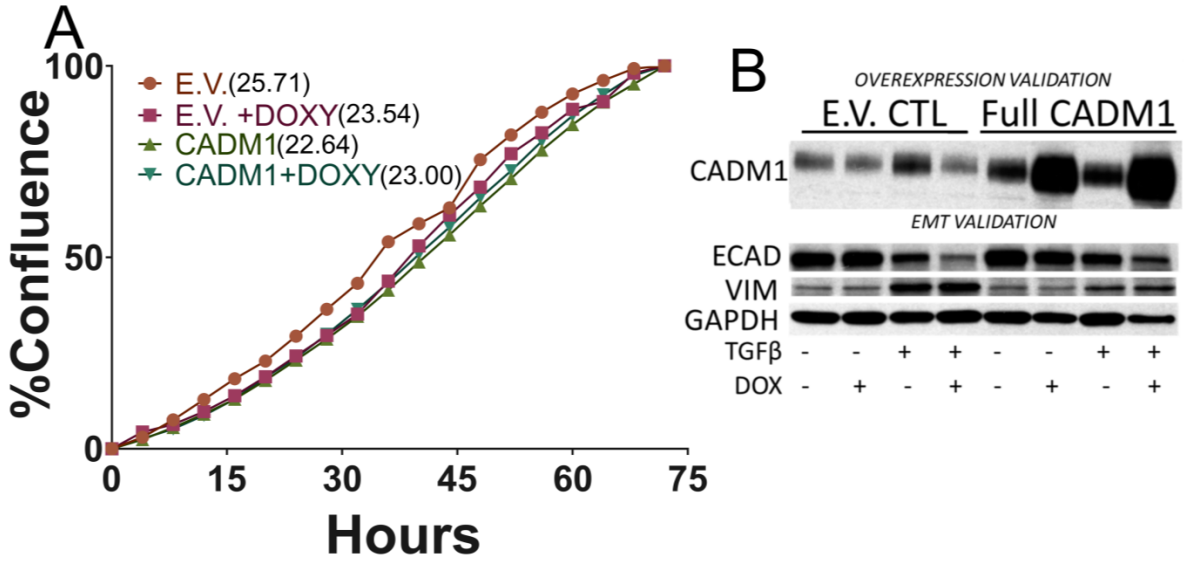

Figure 5-3 CADM1 Overexpression Does Not Effect Cell Growth or EMT

(**A**) Confluence percentage as calculated by an Incucyte Imager, EssenBio INC. of A549 doxycycline-inducible overexpression of CADM1 or with an Empty Vector (E.V.) control.

Doubling times were not significantly different shown in parentheses with or without doxycycline or induced CADM1 expression.
(**B**) Western immunoblot validation of CADM1 overexpression and TGF-β-induced EMT was validated as seen by ECAD loss and Vimentin (VIM) increases.

CADM1 Overexpression and Re-expression Enhances NK Cell Cytotoxicity

However, dox-induced CADM1 overexpression alone was sufficient to confer susceptibility to NK-mediated cytotoxicity, without the induction of EMT (**Figure 5-4A**). Similarly, in a cell line with CADM1 promoter hypermethylation (H1299) (Heller et al., 2006), we were able to restore CADM1 expression by culturing H1299 cells in the presence of 5'-azadeoxycytidine (5-aza), a pan-demethylating agent, for 6 days **(Figure 5-4B**). Consistent with the restored CADM1 expression, 5-aza treatment also rendered H1299 cells susceptible to NK-mediated cytotoxicity (**Figure 5-4B**). In both systems where CADM1 was expressed, we found no A549 cell growth alterations indicating that the tumor suppressive functions are not apparent in two-dimensional culture conditions. This fact and the enhanced NK susceptibility may indicate an *in vivo* only suppressive effect or a NK-mediated effect. In either case, the efficacy of above two methods demonstrates that restoring CADM1 expression can be a potential immuno-therapeutic strategy for lung cancer.

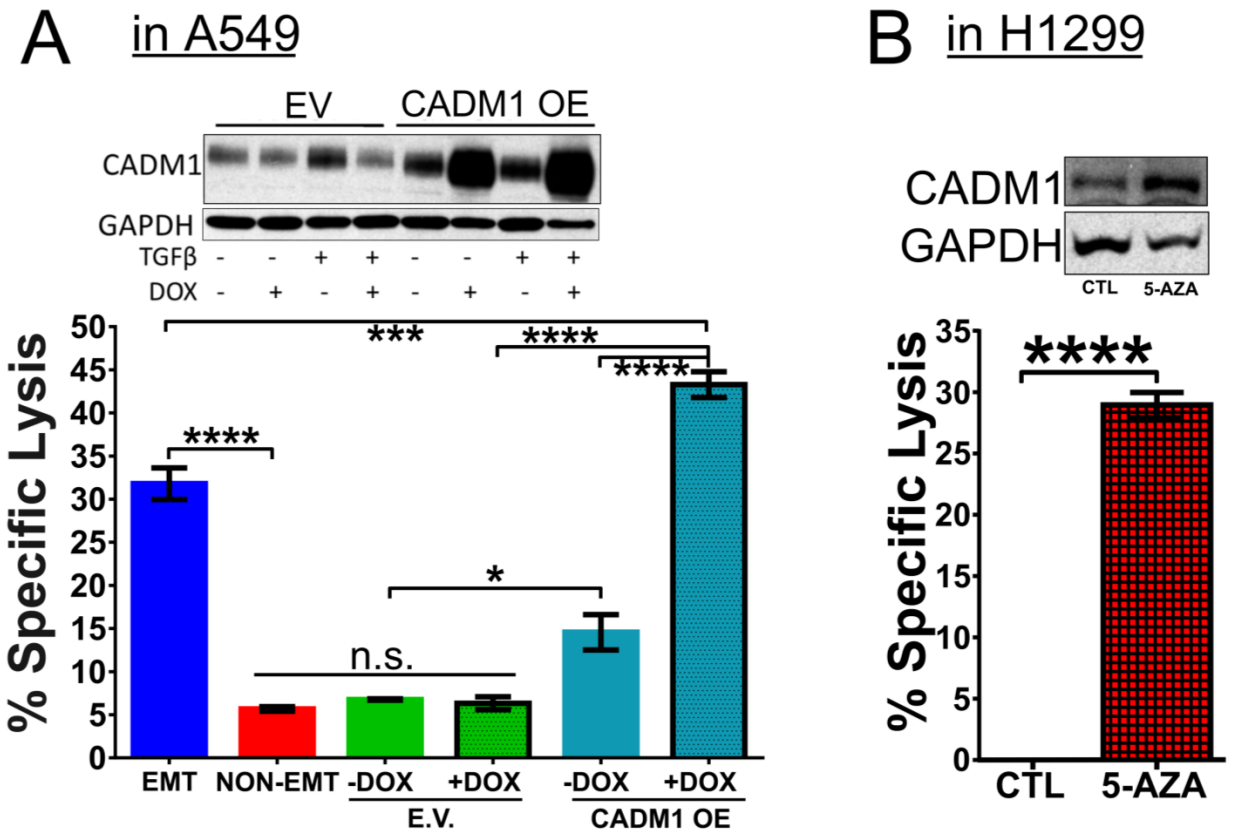

Figure 5-4 Restoring CADM1 Expression in Tumor Cells is Sufficient to Confer Susceptibility to NK Cytotoxicity

(**A**) Stable A549 cell lines expressing empty vector (E.V.) or vector with doxycycline (DOX)-inducible human CADM1 overexpression (CADM1 OE) were developed. Expression of TGFβ-induced CADM1 was assessed in the presence and absence of doxycycline, by western immunoblotting and susceptibility to NK cytotoxicity was assessed by using NK92mi cells as effectors, as described for figure 1. mean±SEM shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001.
(**B**) H1299 cells harboring CADM1 promoter hypermethylation were cultured in the presence and absence of a pan-DNA methylase inhibitor, 5-Azacytidine (5-Aza). CADM1 expression and NK cytotoxicity was assessed as described above. Mean±SEM shown and two-tailed, unpaired, t-tests were performed *P < 0.05, **P < 0.01, ***P < 0.001.

CADM1 Tumor Suppression is NK-Dependent in A549 Tumors

Testing for enhanced cytotoxicity did not fully confirm that NK cell immune surveillance was the critical factor that determined the tumor suppressive nature of CADM1. Further, the lack of growth inhibition does not mean that growth in a three-dimensional culture or *in vivo* would not be affected. To address both questions, we implanted A549 cells described previously into

RAG1$^{-/-}$ mice with and without NK cells. These mice were dosed with doxycycline and treated with either ASGM1 or control rabbit serum 2 days prior to implanted the cells. A549 cells were also pretreated with doxycycline prior to implant. We first assessed primary tumor growth (**Figure 5-5A**) and observed that CADM1 OE A549 cells would grow unimpeded when NK cells were depleted. Consistent with our *in vitro* findings, primary tumors showed a lack of growth rate alteration with CADM1 OE. Tumors were excised to show the disparate tumor sizes achieved (**Figure 5-5B**). Furthermore, CADM1 overexpression did not affect the ability of A549 cells to metastasize in the absence of NK cells as we have shown previously (**Figure 5-5C**). This evidence points towards a requirement for the tumor suppressive nature of CADM1, being that it must be in the presence of an intact NK immune surveillance system.

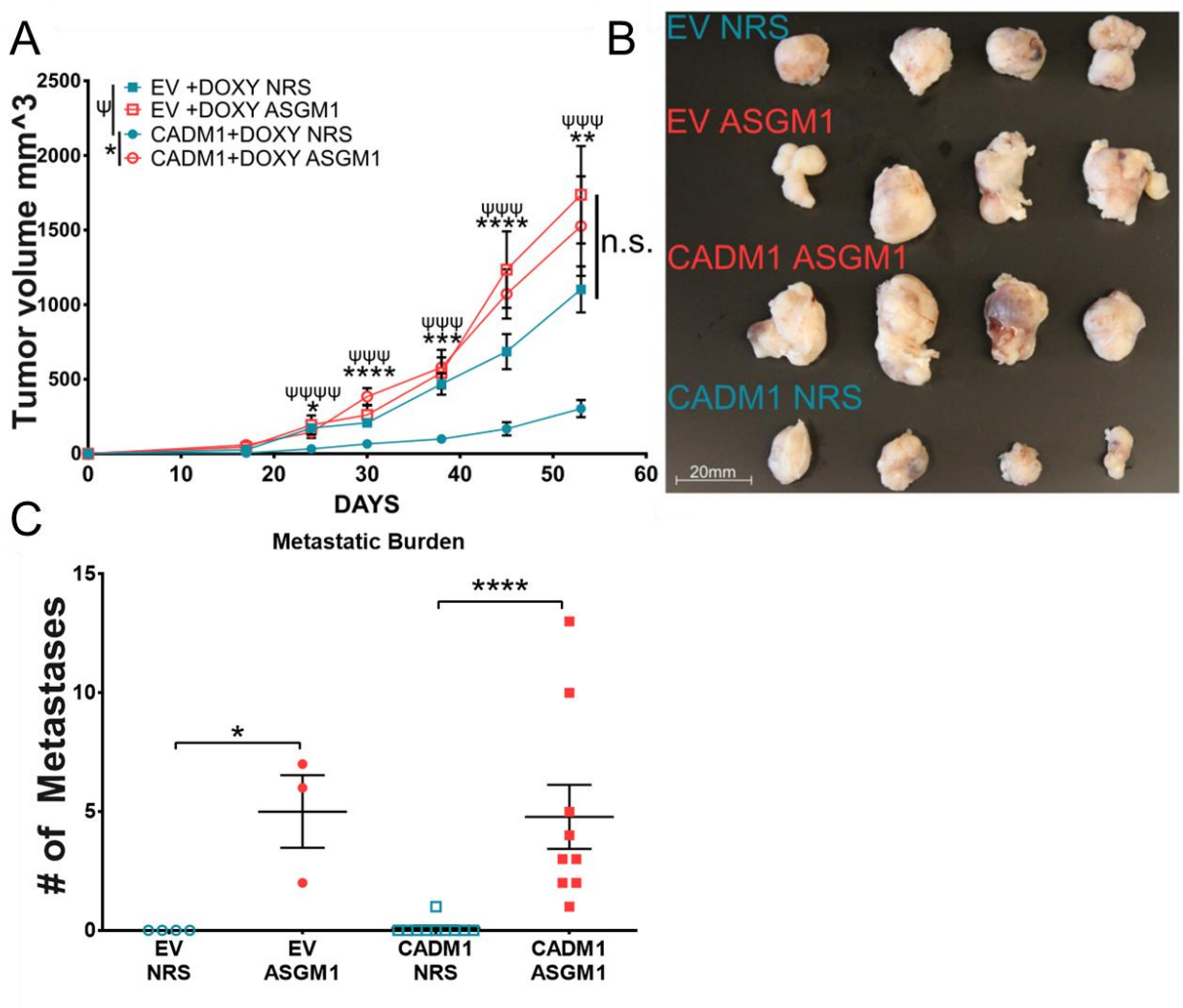

Figure 5-5 Reduced A549 Tumor Burden by CADM1 Overexpression is NK-Dependent

(**A**) To assess the role that CADM1 plays primary tumor growth of A549, cells were implanted in the dorsal flanks of RAG1$^{-/-}$ mice. Mice and cells were pre-treated with doxycycline to induce CADM1 expression. NK cells were depleted with weekly administration of ASGM1. Student's unpaired two-tailed t-tests were performed to determine significance in tumor burden in indicated groups.
(**B**) Gross observation of tumor size is shown, scale bar is 20mm
(**C**) Spontaneous metastatic burden was assessed from the groups in (**A**) Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001.

CADM1 Tumor Suppression is NK-Dependent in H1299 Tumors

We also sought to explore another model of CADM1 OE *in vivo*. It could be possible that the complete removal of CADM1 by the cancer cells could then result in a more tumor intrinsic suppression by CADM1 rather than being dependent on NK cells. The possibility of a certain cancer cell being more sensitive to CADM1 signaling is entirely plausible.

To test this hypothesis we selected H1299 lung cancer cells which have lower expression of CADM1 than in A549 cells (**Figure 5-4B**). Since A549 only has an LOH for CADM1 and not full promoter hypermethylation as in H1299. Instead of using a pan-demethylation agent, we constructed CADM1 overexpressing cell line in the same manner as described previously for A549 and validated over-expression (**Figure 5-6A**). Again, we found no *in vitro* growth defects with over expression. Similar to A549 CADM1 OE *in vivo* primary tumor growth, we also found no differences in growth when NK cells were depleted (**Figure 5-6B**).

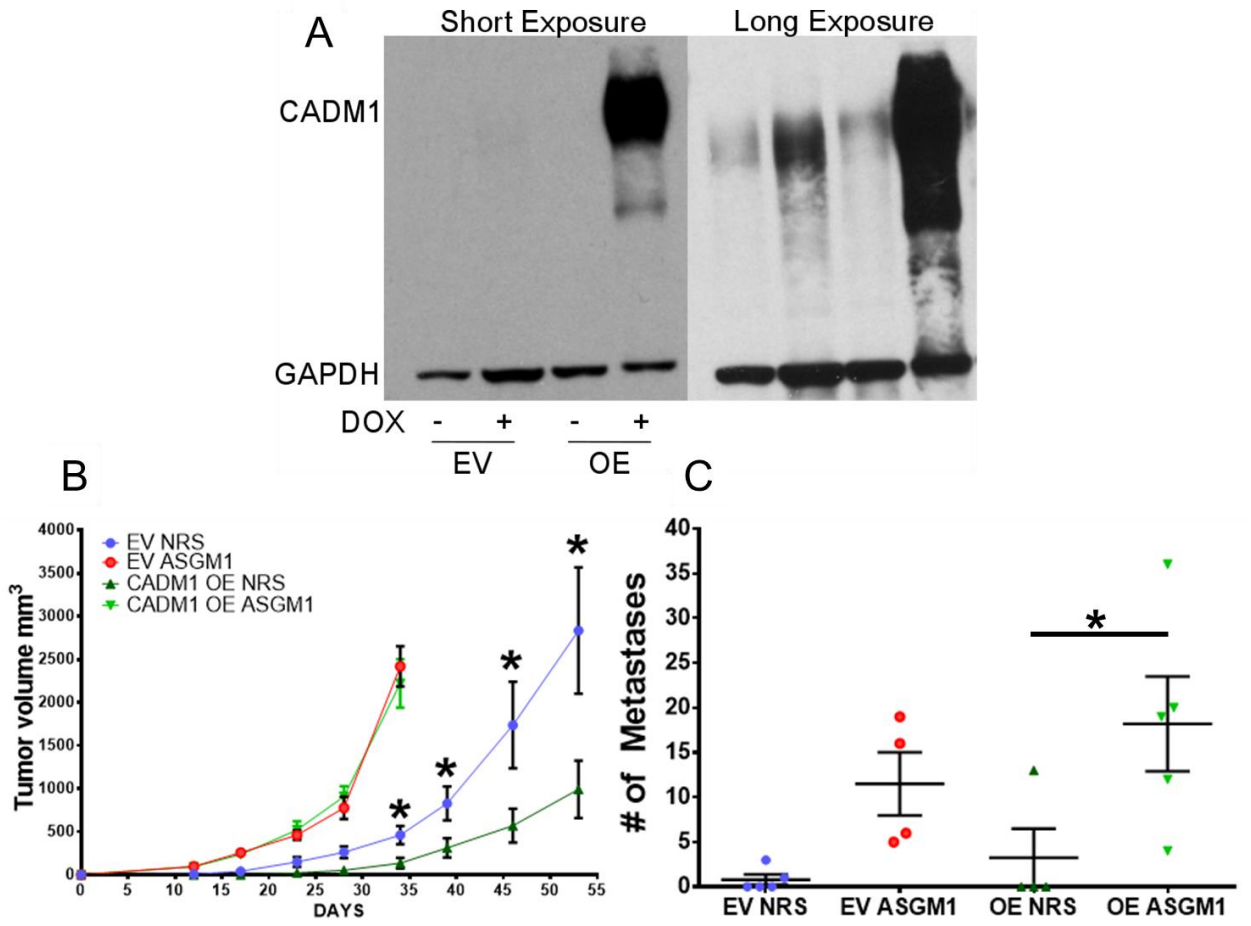

Figure 5-6 Reduced H1299 Tumor Burden by CADM1 Overexpression is NK-Dependent

(**A**) Westernblot analysis of H1299 doxycycline inducible CADM1 expression OE or Empty Vector Control (EV). A short and long exposure where taken to show the extent of overexpression.
(**B**) To assess the role that CADM1 plays primary tumor growth of H1299, cells were implanted in the dorsal flanks of $RAG1^{-/-}$ mice. Mice and cells were pre-treated with doxycycline to induce CADM1 expression. NK cells were depleted with weekly administration of ASGM1. Student's unpaired two-tailed t-tests were performed to determine significance in tumor burden in indicated groups.
(**C**) Spontaneous metastatic burden was assessed from the groups in (**B**) Mean±SEM is shown and one-way ANOVA with Tukey's post-hoc analysis was performed *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001.

Consistently, when NK cells were present CADM1 OE H1299 tumors were smaller than H1299 EV controls (**Figure 5-6B**). Intriguingly, we did observe an NK-dependent growth reduction in H1299 EV cells that did not overexpress CADM1. This suggests that there may be

other activating ligands present on H1299 cells that are triggering a response. Additionally, we found an NK cell-mediated metastasis-specific immune surveillance. Further, CADM1 OE did not affect the ability of H1299 cells to metastasize. In regards to CADM1 and metastasis, both A549 and H1299 showed remarkable similarity.

NK depletion affected the primary tumor growth kinetics and the lack of CADM1 expression in EV controls did not result in metastatic outgrowth. These results suggest that there may yet be other cross reactive activating ligands at play other than CADM1, especially in the case of H1299. Regardless of the disparities in exact experimental observations, we have shown in both models that CADM1 tumor suppression is NK-dependent. We sought to determine how CADM1 is regulated to potentially exploit this signaling to enhance CADM1 expression.

Regulation of CADM1 by TGFβ

CADM1 Cycles with EMT/MET

Given the significant induction of CADM1 expression during EMT, we wanted to understand if CADM1 cycled similarly to ECAD in response to EMT/MET. If CADM1 also cycled in response to EMT/MET it could help shed light on evasive strategies at the distant site of metastasis. We observed a robust modulation of CADM1 protein in response to TGFβ-induced EMT-MET cycling concurrent with the epithelial (ECAD) and mesenchymal markers (VIM) in a time-dependent fashion (**Figure 5-7A**). We further validated TGF-β-induced CADM1 expression with concomitant down regulation of ECAD by immunofluorescence (**Figure 5-7B**).

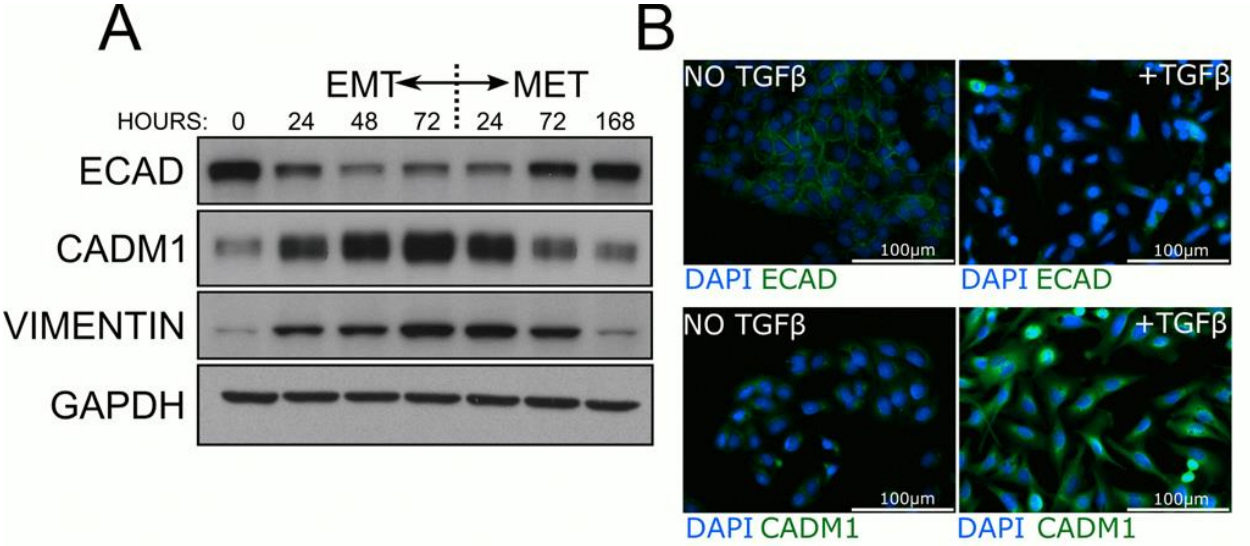

Figure 5-7 CADM1 Cycles with TGFβ-Induced EMT and MET

(**A**) A549 cells were treated with TGFβ (5ng/ml) to induce EMT and total proteins were extracted at the indicated times. After 72hrs, cells were washed thrice and replaced with fresh media to induce MET and total proteins were extracted at the indicated times. Protein expression of ECAD, CADM1, vimentin and GAPDH were assessed by western immunoblotting.
(**B**) A549 cells treated with and without TGF-β for 72hrs were fixed and assessed for ECAD and CADM1 expression by immunofluorescence staining. Scale bars are 100μM.

After determining the critical activating role that CADM1 plays in NK immune surveillance and ECAD's inhibitory role, we sought to determine the cancer cell intrinsic pathway that allows for the TGFβ-induced upregulation of CADM1. We previously have shown that TGFβRI is critical for TGFβ-induced EMT and metastasis *in vivo*, but what remained unanswered is the downstream signaling that occurs to induce CADM1.

CADM1 Induction is Regulated by Canonical TGFβ Signaling via SMAD2/4 Transcription Factors

TGFβ canonically signals through various SMAD proteins. The SMAD protein family consists of 8 members with a group of receptor SMADs (1,2,3, 5, and 8/9), a single co-regulatory SMAD4, and inhibitory SMADs (6 and 7) (Massague et al., 2005). Receptor SMADs directly

signal through the TGFβ receptor complex. Receptor SMADs then bind with SMAD4 and translocate to the nucleus and subsequently bind to DNA occasionally with the help of a DNA-binding proteins and initiate gene transcription of their respective target genes (Massague, 2012).

Given the robust induction of CADM1 by TGFβ in A549 cells, we began to dissect the specific components of the TGFβ signaling pathway. SMAD2/3/4 stable cell lines were selected by knockdown efficiencies shown in (**Figure APP 5**). Intriguingly, we found that CRISPR-Cas9-mediated deletion of SMAD2 had no effect on EMT, but blocked induction of CADM1. SMAD3 or SMAD4 deletion partially blocked TGFβ-induced EMT as shown by ECAD knockdown. CADM1 upregulation was unaffected in SMAD3 deletion (**Figure 5-8**). SMAD2 and SMAD4 deletion resulted in abrogated CADM1 induction consistently given that SMAD2 requires SMAD4 for DNA binding. Densitometry for the various levels can be found in (**Figure APP 6)** Taken together, this data suggests that CADM1 is regulated by SMAD2 and SMAD4 in an EMT-independent manner.

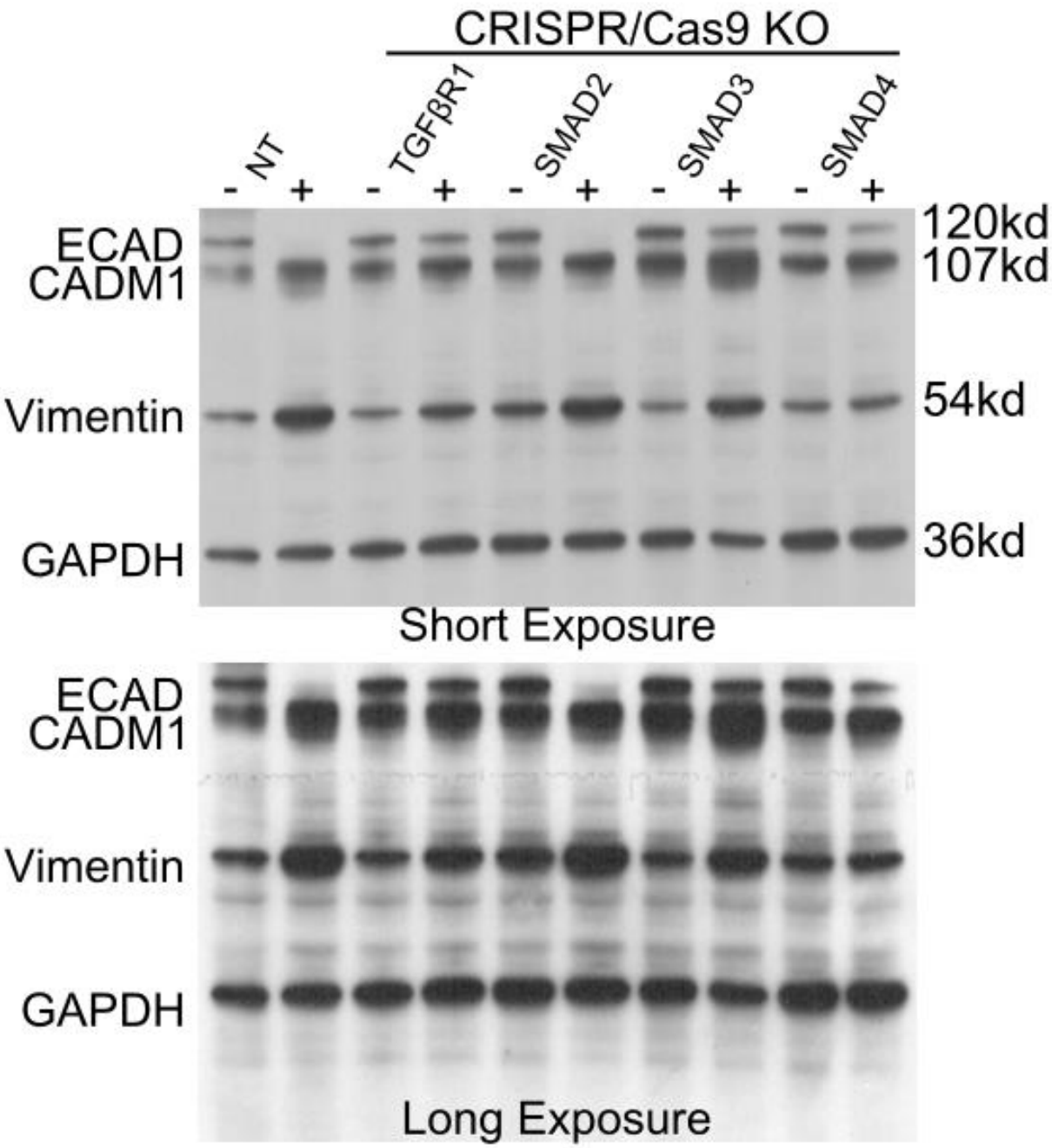

Figure 5-8 CADM1 Regulation in the Canonical TGFβ Signaling Pathway

Utilizing CRISPR/Cas9-mediated deletion of the indicated genes to form stable A549 cell lines. These cell lines were then subjected to TGFβ-induced EMT for 72hrs. ECAD, CADM1, Vimentin, and GAPDH were probed via western blot. Shown are two separate exposures revealing varying aspects of the relative levels of protein expression.

CADM1 Expression in Primary Tumor Correlates with Improved Patient Survival and Decreased Metastasis

To further demonstrate the potential clinical significance of CADM1, gene expression of *CADM1* was assessed in a primary human lung adenocarcinoma dataset (n=442) (Shedden et al., 2008). Univariate Cox overall survival analysis revealed increased expression of *CADM1* in lung tumors strongly correlated with prolonged patient survival (**Figure 5-9A**). *CADM1* expression inversely correlated with higher tumor stage and positive nodal status (**Figure 5-9B-C**). We further validated the survival analysis in an independent lung adenocarcinoma data set (n=720) (**Figure 5-9D**)(Gyorffy et al., 2013), and also in a ER+ breast cancer data set (n=548) (**Figure 5-9E**) (Gyorffy et al., 2010), as we observed an enhanced EMT-induced NK cytotoxicity in MCF7, an ER+ breast cancer cell line (**Figure 2-3**). In all tumor types, we observed greater survival with increasing *CADM1* expression. Additionally, we investigated the correlation between *ECAD* expression and patient survival in the same cohorts and found no effect on patient outcome, (**Figure 5-10**). This observation is consistent with the modest effect of ECAD/KLRG1 inhibition (**Figure 4-2**) on NK cytotoxicity, indicating the need of an activating ligand for optimum NK activation.

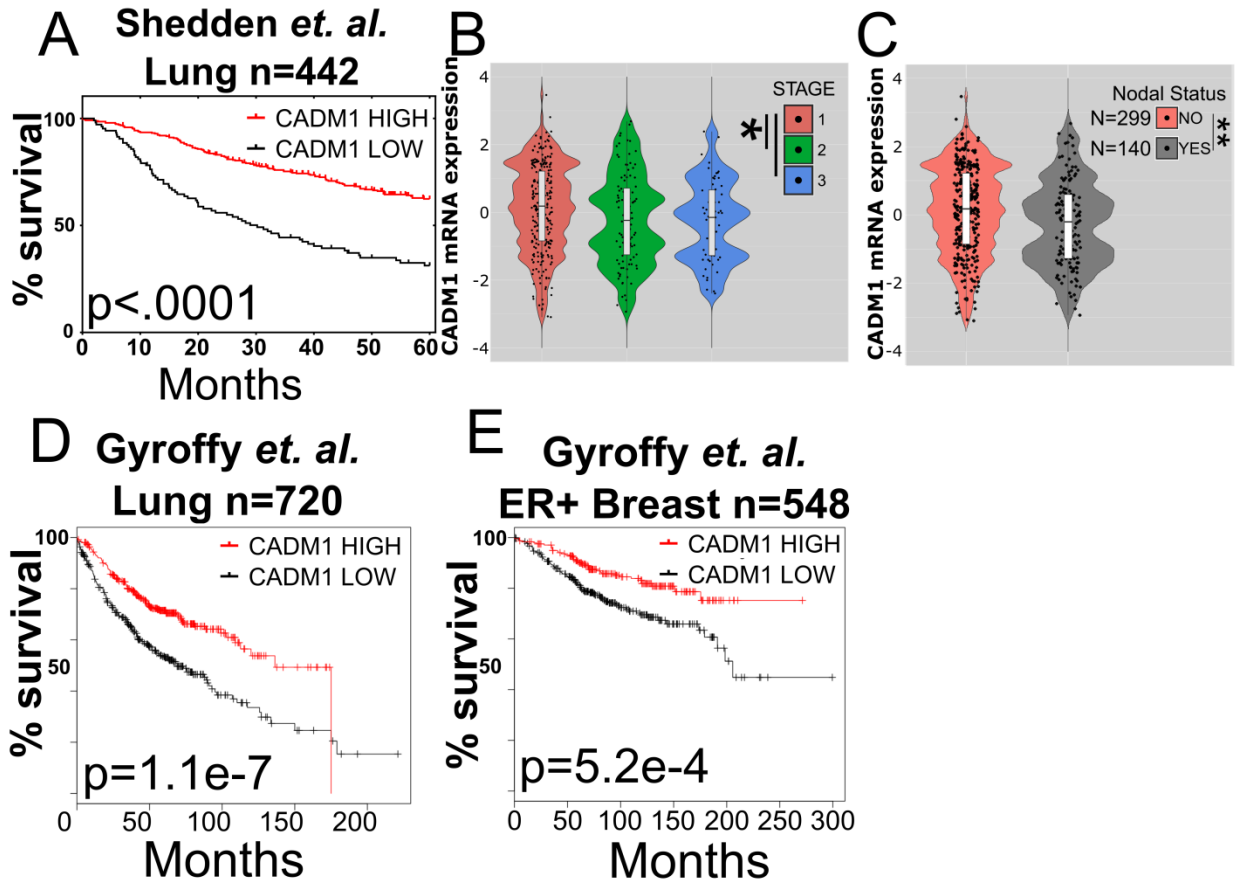

Figure 5-9 Reduced *CADM1* Expression Correlates to Worse Patient Survival and Metastasis

(**A**) Lung adenocarcinoma patient cohort, Shedden et al.,-lung (n=442), was stratified into median low and high *CADM1* expressing groups and assessed for overall survival.
(**B** and **C**) Shedden et. al. data set is further classified into sub-groups based on tumor stage and nodal status recorded at the time of diagnosis. Mean *CADM1* expression and its distribution are depicted in the violin plots with box and whisker overlays in white. Two-tailed, unpaired, t-tests were performed.
(**D**) Lung adenocarcinoma patient cohort, Gyroffy et al. –lung (n=720), was stratified into median low and high *CADM1* expressing groups and assessed for overall survival.
(**E**) Breast carcinoma patient cohort, Gyroffy et al.,-ER+ breast (n=548), was stratified into median low and high *CADM1* expressing groups and assessed for overall survival. (**A,D,** and **E**) Datasets shown here are Kaplan-Meier survival curves with Log-Rank p-values comparing the groups.

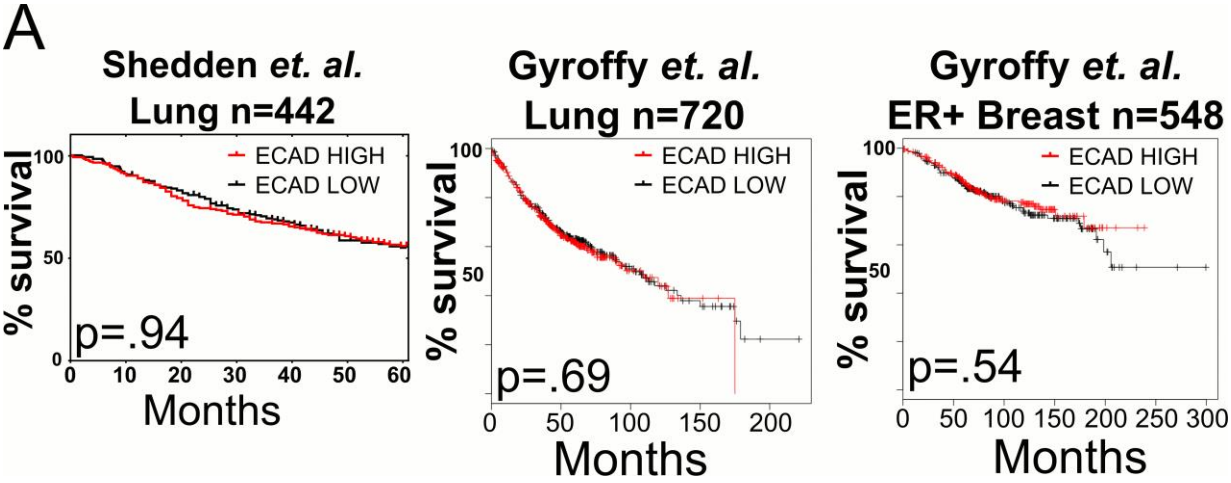

Figure 5-10 E-Cadherin expression in Patient Cohorts does not reveal survival benefit

(**A**) Lung adenocarcinoma patient cohort, Shedden et al.,-lung (n=442), was stratified into median low and high *ECAD* expressing groups and assessed for overall survival. Lung adenocarcinoma patient cohort, Gyroffy et al. –lung (n=720), was stratified into median low and high *ECAD* expressing groups and assessed for overall survival. Breast carcinoma patient cohort, Gyroffy et al.,-ER+ breast (n=548), was stratified into median low and high *ECAD* expressing groups and assessed for overall survival. Datasets shown here are Kaplan-Meier survival curves with Log-Rank p-values comparing the groups.

NK Cells Control *De Novo* Oncogenesis

The patient cohort and human cell line analyses supported our hypothesis that CADM1 is important in patient survival and metastasis, putatively due to NK immune surveillance. We next sought to explore a scenario whereby NK cells would not be present during oncogenesis and tumor progression, we would then assess for CADM1 modulations in these cancers. We elected to use a robust model whereby the addition of CRE to a loxP system can induce autochthonous cancer in mice (Rhim et al., 2012). Cancer can be driven by using a tissue specific gene with a linked CRE gene or by the addition of a CRE-expressing virus. Our model consists of a globally-expressing floxed TP53, stop floxed G12D mutant KRAS, and a YFP reporter. Cells that encounter CRE will then overexpress mutant KRAS, be null for TP53, and express YFP has a reporter and readout of CRE activity (**Figure 5-11**).

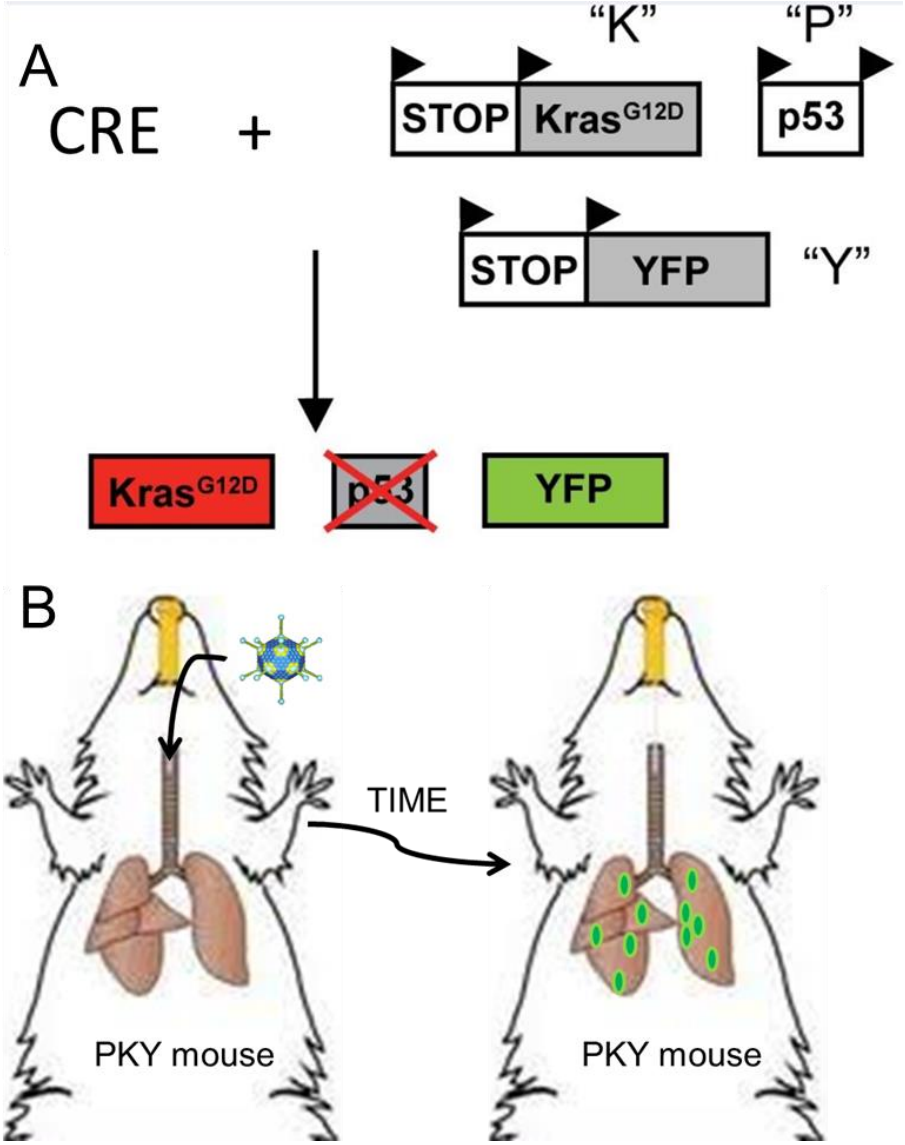

Figure 5-11 Genetics of PKY Murine Models of *De Novo* Lung Cancer

(**A**) Genetic schematic overview of the mouse model used showing the flox sites shown in black triangles and the outcome post-CRE activity, figured modified from (Rhim et al., 2012).
(**B**) Methodology utilized inducing lung-specific cancer. Mice were instilled intratracheally with adenovirus expressing CRE-recombinase. Mice were monitored over 90 days and lungs were excised and burden was quantified by an independent pathologist.

There is typically a 50% rate of distant metastatic disease in this model of lung cancer which is consistent with that observed in lung cancer patients. We cannot determine lobe-to-lobe

metastasis due to the nature of the CRE-expressing virus instillation. We hypothesized that NK cell depletion would increase metastatic burden in these mice and allow for CADM1 expression.

NK Cells Control De Novo Tumor Burden and Metastatic Spread

To fully determine the role of NK cell involvement we first depleted NK cells using anti-NK1.1 as described previously. Two days later we performed intra-tracheal instillation of $2.5x10^7$ adenovirus expressing CRE-recombinase particles. Mice were then monitored for survival and continually depleted of NK cells every 5 days. After 90 days had elapsed since CRE instillation, all NK-depleted mice, but only 50% of control mice had died of their tumor burden (**Figure 5-12A**). Excised lungs were fixed, sectioned, and H&E stained for pathological scoring. In agreement with the survival data NK-depleted mice had twice has many lung nodules (**Figure 5-12B-C**). Three examples from each group can be seen in (**Figure 5-13**). Further, there was a marked increase in high-grade tumors in mice depleted of NK cells. This was unexpected as we have previously found no involvement of NK cell surveillance on primary tumor implants.

Additionally, NK-depleted mice had significantly more metastatic burden as shown in (**Figure 5-14**). Large masses formed where mediastinal lymph nodes used to be and in some cases extra-thoracic masses were found as well. Consistently, we also observed mediastinal masses in control non-depleted mice as well, but were fewer and smaller. We next wanted to explore if the primary tumors or the metastases had altered CADM1 or ECAD expression.

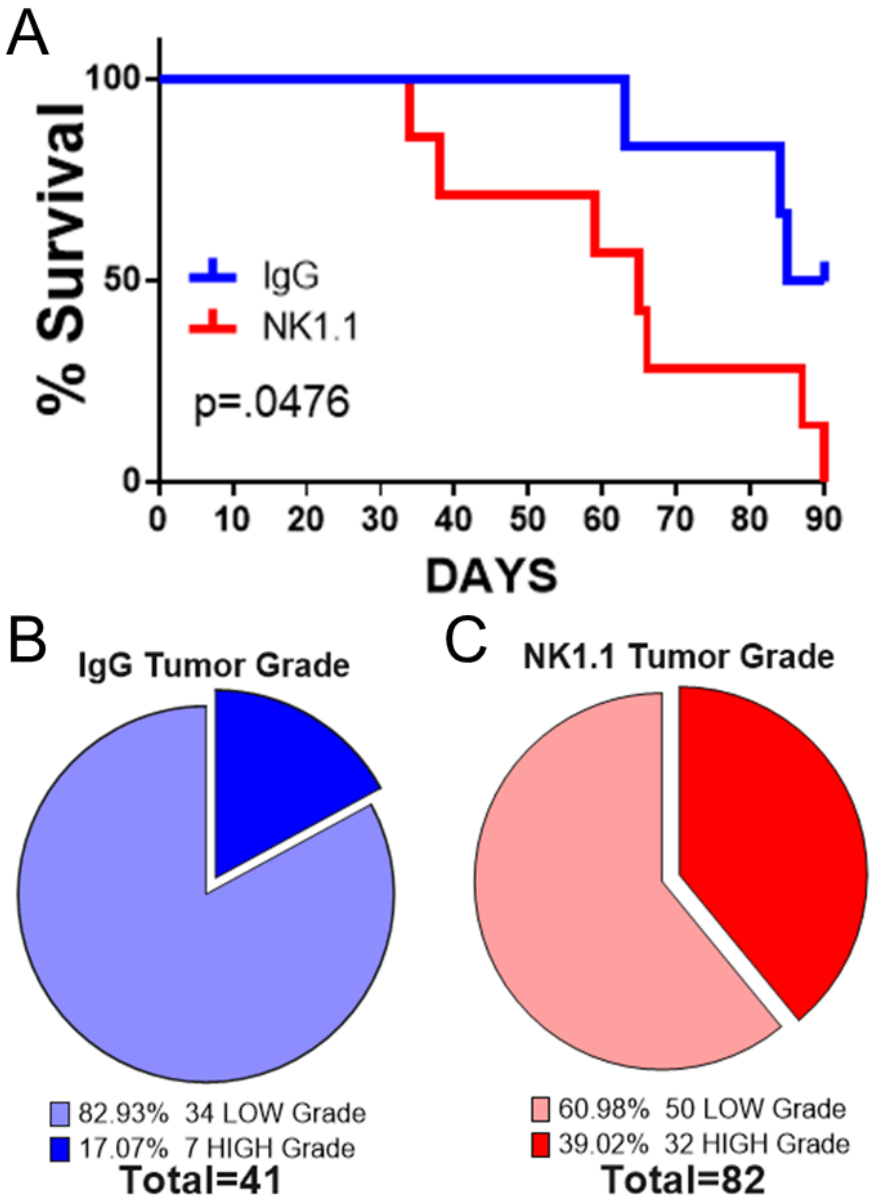

Figure 5-12 NK cells Protect Against Genetically-Induced Cancer in PKY Mice

(**A**) TP53 null, KRAS mutant, and Yellow Fluorescent Protein CRE-inducible (PKY) mice were depleted of NK cells or using 200μg of anti-NK1.1 or a control antibody every 5 days and assessed for overall survival. Shown is a Kaplan-Meier survival curves with Log-Rank p-values comparing the groups.
(**B-C**) Lungs of mice were excised and fixed upon death, cyrosectioned and H&E stained, examples are shown in (**Figure 5-13**). These sections were sent to an independent pathologist for tumor burden quantification. Pie charts detail the number of total nodules and the distribution of low and high-grade tumors.

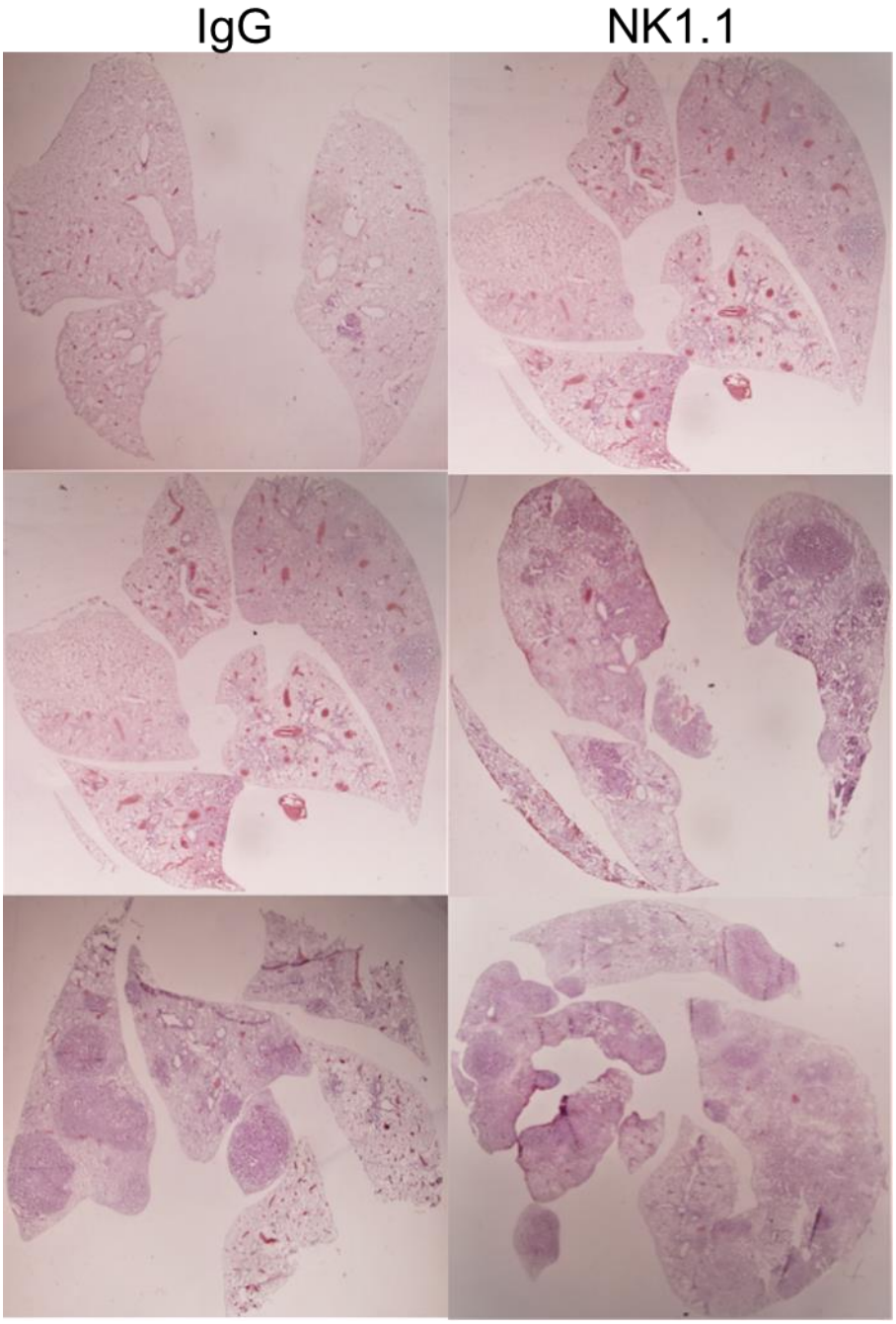

Figure 5-13 Murine PKY Lung Cancer Model Histology

Three examples of H&E lung sections from each PKY mouse group.

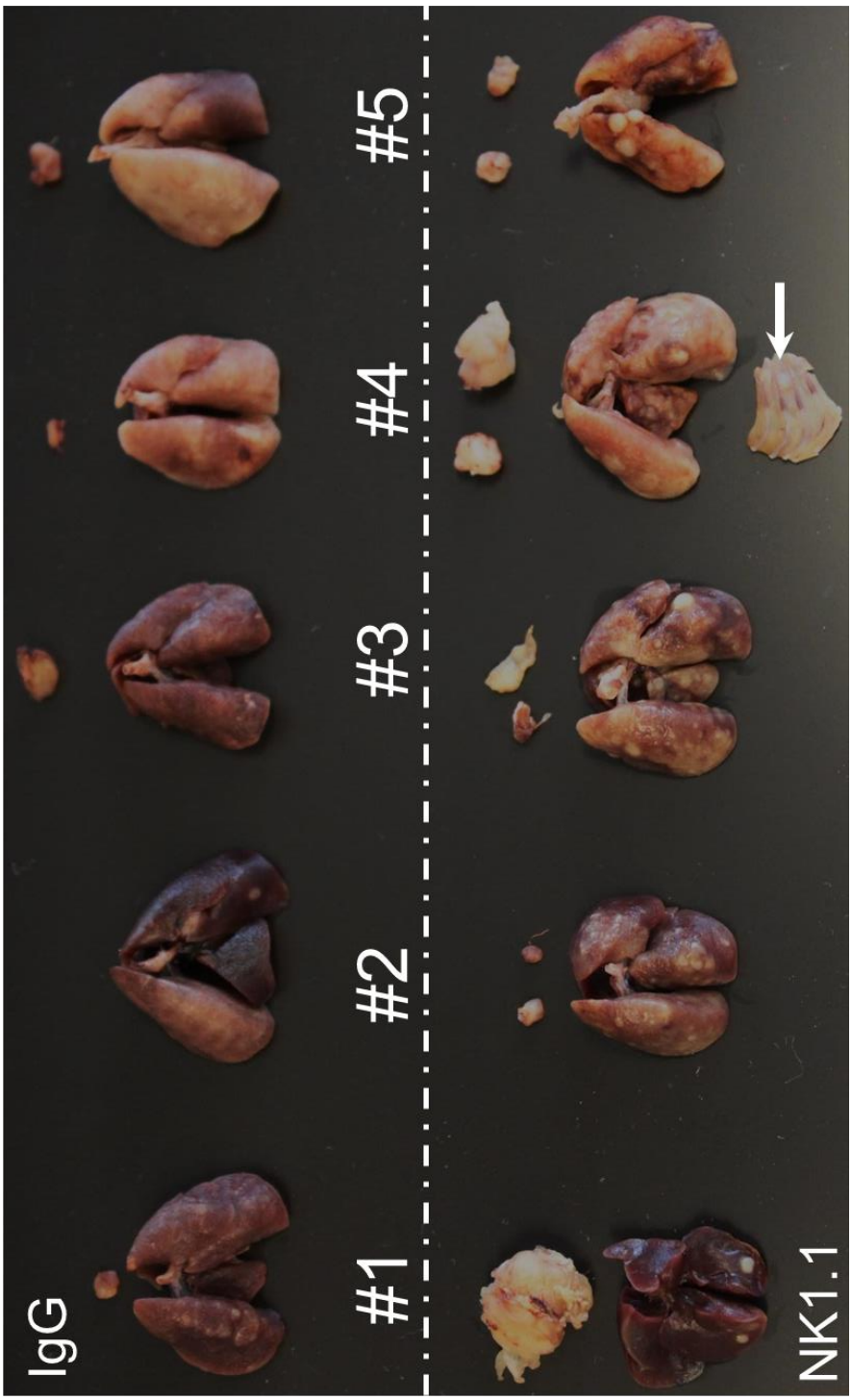

Figure 5-14 Gross Anatomy of Lung Tumor Burden and Metastases of PKY Mice

Gross anatomy of 5 mice from each group of PKY mouse is shown. Metastatic nodules are above the lungs and overt lung tumor burden is apparent. The white arrow indicates a metastatic nodule that was between the ribs of that mouse.

PKY Mouse-Derived Cell Line Analysis

We isolated fresh tissue from two mice, one from each group IgG and NK1.1. Next, we isolated and cultivated some tumor cell lines from these mice. Upon dissection, mincing, and enzymatic digestion; primary and metastatic tumor nodules were plated on mitomycinC inactivated fibroblast cell line 3T3 with Rho-associated protein kinase (ROCK) inhibitor added. ROCK inhibition allows for proliferation and survival of isolated cells from tissues (Liu et al., 2012). Eventually, large YFP tumor cell colonies began to form (**Figure 5-15A**). Utilizing differential trypsinization and re-plating cells without fibroblasts, we attained pure murine YFP-positive tumor cells lines verified visually and with flow cytometry. We could then maintain these cell lines in the absence of fibroblasts and ROCK inhibitor after 4 weeks and continually thereafter.

These 4 cells lines were then analyzed for ECAD and CADM1 expression via western blot (**Figure 5-15B**). Consistently, both primary tumor cell lines had similar ECAD and CADM1 levels. The metastases isolated from the NK sufficient mouse had significantly -reduced CADM1 levels compared to any other cell line. This loss of CADM1 in the presence of NK cells is indicative of an immune evasive strategy employed by cancer cells. This line of evidence further supports our human cell line analysis (**Figure APP 4**), where we lacked a matched primary and metastatic outgrowth. Further investigation into the role of CADM1 during oncogenesis is imperative.

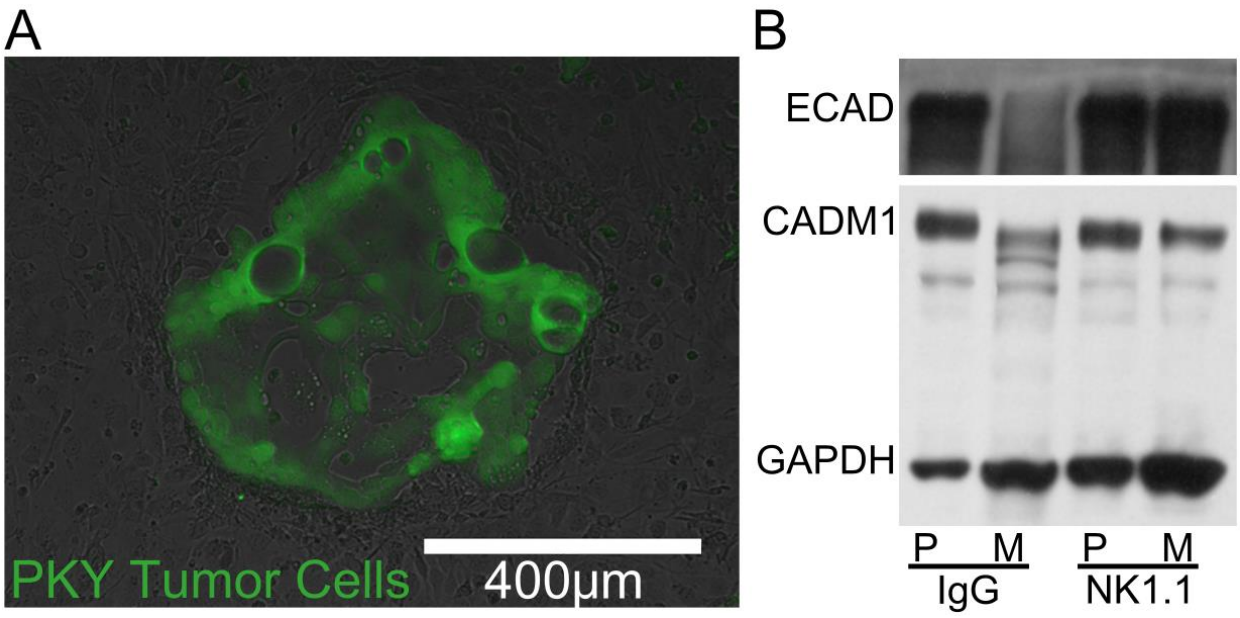

Figure 5-15 Isolated Primary Tumor and Metastatic Cell Lines from PKY Mice

(**A**) Upon death, PKY mice of both NK replete and deleted animals had pieces of tumor mass excised and grown in culture with fibroblast feeder cells. Scale bar is 400µm. Tumor cells are YFP positive, but appear green due to excitation filters.
(**B**) Western immunoblot analysis of ECAD, CADM1, and control GAPDH of 4 cell lines created from these de novo induced PKY cancers. P=primary lung tumor, M=Metastatic site. These are paired samples from a single mouse in each group.

Chapter 6 Discussion

EMT, Metastasis, and NK Immune Surveillance

Studies thus far have focused on understanding how a small proportion of disseminating cells escape host surveillance and metastasize. Unfortunately, very little attention is paid to the understanding of the mechanisms which successfully eradicate more than 99% of tumor cells. It is now increasingly appreciated that the immune system plays an important role in the surveillance against metastasis (Hong et al., 2010; Olkhanud et al., 2009; Paolino et al., 2014; Slaney et al., 2013; Yang et al., 2008). Since EMT is critical for metastasis, exclusive focus on evasive or resistance mechanisms that cells acquire after EMT may have promoted an unintended bias; that cells undergoing EMT must be resistant to host anti-tumor responses. On the contrary, it is equally feasible that metastatic cells after EMT are also vulnerable to host immune surveillance. In other words, when cancer cells exit the immunosuppressive primary tumor microenvironment it is possible that they may pay a toll to metastasize by becoming more susceptible to host immune surveillance.

Strongly supporting this notion, our studies show that in addition to promoting an invasive phenotype (Keshamouni et al., 2006; Reka et al., 2011; Reka et al., 2010), EMT renders cancer cells more susceptible to NK-mediated killing, *in vitro*, by modulating activating and inhibitory ligands. This is consistent with a singular *in vitro* study that showed increased susceptibility of a colon cancer cell line to NK cells post-EMT (Lopez-Soto et al., 2013). More importantly, for the first time, we demonstrated the consequence of this enhanced susceptibility on tumor metastasis *in vivo* in multiple mouse models. In addition, we observed this

phenomenon in a breast cancer cell line, highlighting the broader significance of this study. Together, this suggests that the NK cell-mediated immune surveillance mechanism may contribute, in part, to the inefficiency of the metastatic process.

To date, a number of studies in mouse models have implicated NK cells in the control of metastasis (Lopez-Soto et al., 2017) and also showed that evasion of NK-mediated immune surveillance plays a critical role in maintaining metastatic latency (Malladi et al., 2016). Supporting these observations, clinical studies in multiple solid tumors have demonstrated an inverse correlation between the number of circulating or tumor-infiltrating NK cells and the presence of metastasis at the time of diagnosis (Lopez-Soto et al., 2017). Similarly, higher expression of NK cell activating receptors has been shown to correlate with better prognosis in patients with or at the risk of metastasis. However, mechanisms involved in this metastasis-specific control are unknown. Our current observations are not only consistent with the above notion but also provide a mechanistic basis by elucidating the NK activating and inhibitory axis involved.

Moreover, we did not observe any effect of NK cell depletion on the growth of the epithelial-like primary tumors, with the exception of H1299. TGFβ is known to inhibit NK cell function (Ghiringhelli et al., 2005; Viel et al., 2016) and the TGFβ-rich tumor microenvironment may explain why NK cell depletion has no effect on the primary tumor growth. However, TGFβ levels in circulation or at the site of metastasis are substantially lower than in the primary tumor microenvironment, enabling metastasis-specific immune surveillance. An earlier study demonstrated that EMT allows cancer cells to escape T cell immune surveillance (Akalay et al., 2013) and enable them to leave the primary tumor. In contrast, our data suggests that concomitant modulation of NK ligands that occurs during EMT will make them vulnerable to

NK-mediated cytotoxicity as they abandon the immunosuppressive primary tumor microenvironment, thereby, allowing metastasis-specific control.

Mechanistic Insights of NK-Mediated Metastatic Control

Interestingly, we did not find a role for NKG2D ligands in mediating NK cytotoxicity towards A549 cancer cells. This is despite existing data that suggests EMT promotes NKG2DL-dependent enhanced cytotoxicity in a colon cancer model *in vitro* (Lopez-Soto et al., 2013). The data shown in this study was transformed twice via normalization and shows minimal, but significant, changes in cytotoxicity. Our data shows no effects with two distinct methods to assess NKG2D inhibition *in vitro*. Additionally, our *in vivo* results are consistent with a lack of NKG2D receptor engagement. This is due to the fact that murine NKG2D does not interact with human NKG2D ligands. Given that we have shown human A549 cells that have under gone EMT in mice being targeted it is likely to be a conserved activating and inhibitory axes between species.

ECAD is 81% homologous between humans and mice on the protein level. The direct interaction between KLRG1 and ECAD has been established previously in engineered models of leukemia with forced expression of ECAD. KLRG1 is implicated in the recognition of non-MHC ligand-mediated inhibitory signaling in NK cells (Ito et al., 2006; Schwartzkopff et al., 2007). Even though the role of this interaction has been proposed in tumor progression, it has never been demonstrated in a physiologically-relevant context until this study. Our data suggests that the ratio between the ECAD and the CADM1 expression may dictate the susceptibility of tumor cells to NK-mediated cytotoxicity. Strikingly, CADM1 is 98% and CRTAM is 99-100% identical between species on the protein level, as determined by BLAST alignment of known

protein sequences. This evidence further corroborates the experimental results of CADM1 targeting *in vivo*.

A Novel Tumor Suppressive Function of CADM1

CADM1 was first identified and described as a tumor suppressor gene in 2001 by the Murakami lab, initially named Tumor Suppressor of Lung Cancer 1. It was so named due to the fact that upon adding a yeast artificial chromosomal (YAC) fragment containing a region on the 11q23.2 chromosomal arm back into A549 cancer cells, tumor formation was reduced in nude mice (Kuramochi et al., 2001). Subsequent truncation of the YAC fragment validated that CADM1 was the critical portion driving reduced tumor growth. CADM1 has been shown to be able to form both cis- and trans- homodimerization along with heterotypic interactions with other members of the nectin and nectin-like proteins (**Figure 6-1, Figure 1-4**).

In the case of CADM1, its tumor suppressor activity has been attributed to its cell-cell adhesion functions through homophilic N-terminal interactions. This adhesion allows for signal transduction mediated by its C-terminal intracellular domain and downstream membrane associated guanylate kinases (MaGUK) (Masuda et al., 2002; Murakami et al., 2014). The intracellular domain also binds to the actin cytoskeleton through adaptor proteins that are frequently co-repressed with CADM1 (Heller et al., 2007; Murakami, 2005; Takahashi et al., 2012; Yageta et al., 2002). PI3K has been determined to be a target of CADM1 MaGUK signaling. It is hypothesized that signaling that occurs upon sufficient CADM1 binding results in reduced growth and motility (Murakami et al., 2014). This has been extrapolated to suggest that a dense tumor mass would also be inhibited by CADM1 interactions between cells and slow or halt tumor growth and progression. ECAD has been suggested as a tumor suppressor in a similar

manner by forming strong cell-cell interactions and reducing tumor progression (Pecina-Slaus, 2003).

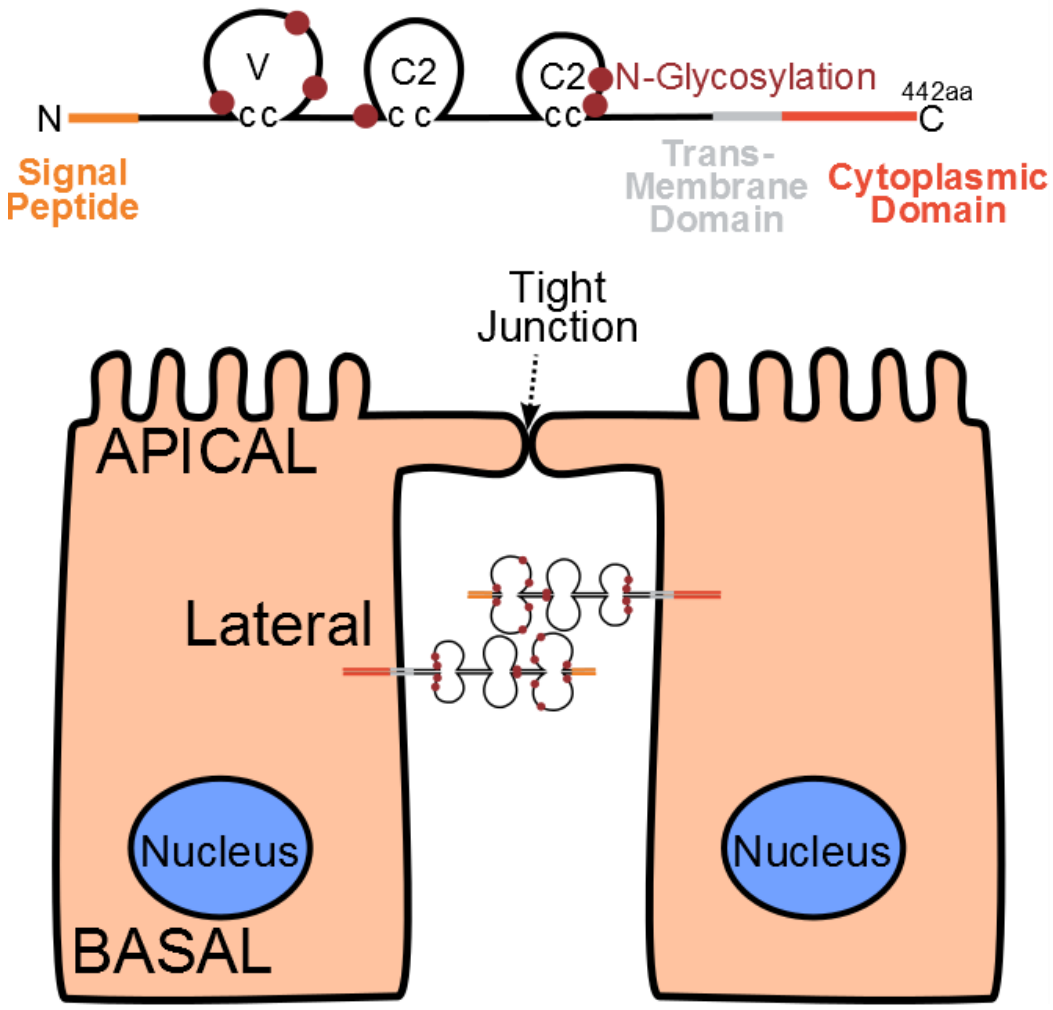

Figure 6-1 Molecular Structure of CADM1 and Cellular Location

A schematic showing the protein domains and glycosylation sites of CADM1. A cartoon showing the binding and location of CADM1 between epithelial cells.

Our work, in conjunction with indirect evidence from other studies, has shown that overexpression has not yielded reduced *in vitro* growth (Faraji et al., 2012; Kuramochi et al., 2001; Seong et al., 2017). There is one example of CADM1 overexpression affecting growth *in vitro*, but the methodology employed was at exceptionally high multiplicity of infection to force

overexpression of CADM1 (Mao et al., 2004). We have overexpressed CADM1 in A549 and H1299 and in both cases found no alterations in growth *in vitro*. Further, we have induced expression via TGFβ or pan-demethylation agents and found no significant alterations. Despite a dearth of *in vitro* evidence to suggest tumor suppression by CADM1, there was *in vivo* evidence strongly supporting the role of CADM1 in tumor suppression. Further, investigations into primary tumors and cell lines from various cancers revealed that many cancers hypermethylate the promoters for CADM1 and/or have a LOH for CADM1 (**Figure 6-2**). Intriguingly, H1299 was isolated from the lymph node metastasis and is hypermethylated for CADM1. This correlative evidence helps solidify the notion of CADM1 loss as a tumor evasive mechanism.

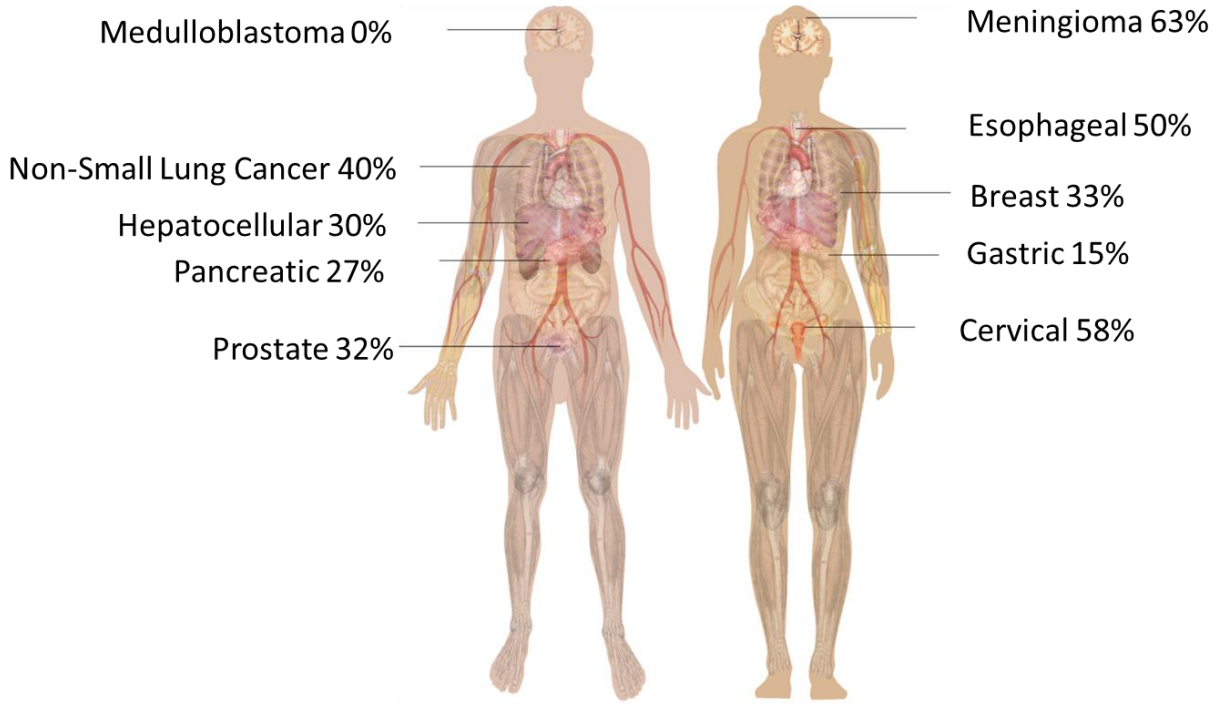

Figure 6-2 CADM1 Hypermethylation Rates in Various Cancers

Data from (Murakami, 2005) Various rates of CADM1 promotor hypermethylation in solid tumors.

Nearly all of the *in vivo* evidence gathered since the year 2001 regarding CADM1 has neglected to account for a critical component of tumor progression: the immune system. The role of CADM1 in the evasion of NK immune surveillance has never been considered as potential mechanism for tumor suppression. Earlier studies that demonstrated the inhibition of tumor growth by CADM1 overexpression were carried out in nude mice or mice that carry functional NK cells and overlooked the role of NK cells in the observed tumor suppression (Faraji et al., 2012; Kuramochi et al., 2001; Mao et al., 2004; Seong et al., 2017).

Our data clearly demonstrates that CADM1 overexpression is sufficient to make cancer cells sensitive to NK cells, *in vitro*. Therefore, assessment of tumor progression in CADM1 overexpressing cells in the presence and absence of NK cells may clarify the mechanism by which CADM1 exerts its tumor suppressive activity. We have shown in two *in vivo* models in which NK immune surveillance is responsible for the tumor suppressive capabilities exhibited by CADM1. Further, our data is consistent with all previously published work regarding CADM1 tumor suppression *in vivo*. Our work specifically highlights the need to explore other signaling paradigms in the context of cancers, specifically adhesion molecules.

Using CADM1 Expression as a Prognostic Factor for Patients

CADM1 has been investigated in a cancer cell intrinsic manner since 2001, which has yielded a wealth of knowledge regarding patient prognosis. Here we have shown that *CADM1* expression is correlated to increased patient survival in three patient cohorts and reduced metastasis in one. Additionally, there have been other studies indicating exceptional survival rates of neuroblastoma patients when *CADM1* is highly expressed (Seong et al., 2017). A number of studies have shown that many cancer types reduce or remove CADM1 (**Figure 6-2**).

We further explored a role for NK cells in modulating tumor progression and tumorigenesis. Our findings further corroborated patient data by revealing that CADM1 is reduced at metastatic sites in mice with NK cells compared to the matched primary tumor cell line or tumor cell lines from mice deficient of NK cells. In addition to our studies, mice that lack CADM1 have increased rates of spontaneous and radiation-induced tumorigenesis and reduced survival (van der Weyden et al., 2012).

Identifying patients that are CADM1 positive may provide insights into treatment options or prognosis. We have shown two methods for CADM1 re-expression and the resultant tumor reducing benefits. Further, an intratumoral viral injection therapy for tumor bearing mice with forced CADM1 re-expression showed tumor reduction as well (Mao et al., 2004). These data are the beginnings of future therapeutic strategies aimed at CADM1 low patient populations. Increasing CADM1 or merely making NK cells more responsive to CADM1 positive tumors is another avenue to explore as even some CADM1 high expressing patients metastasize and succumb to their disease.

Final Thoughts

Limitations and Shortcomings

One limitation of our study is we still do not know at which step(s) of the metastatic cascade NK cells operate to control metastases. To address this, it is important to investigate the effect of NK cell depletion on various steps of metastatic cascade including, local invasion, intravasation, CTC persistence, extravasation and survival at the distant site. Organ-specific deletion or inhibition would provide increased detail of the precise location where NK cells are

exerting their anti-metastatic effects. Animal models that allow for the precise monitoring of each of the above steps are essential to adequately address the above question.

An unfortunate reality of NK depletion strategies is that a purely NK-specific depletion method does not yet exist, as both antibody depletions alter other cellular populations. We have additional unpublished data utilizing SCIDx*beige* mice with A549 tumor cell implantation. These mice lack T and B cells and have cytolytically dysfunctional NK cells. These mice developed spontaneous metastasis of A549 cells similarly to NK depletion experiments shown here. Though our models strongly suggest it is solely classical NK cells responsible for metastatic control, we cannot fully eliminate the possibility of other populations aiding in some way or another. Furthermore, a precise NK-specific knockout of the CADM1 receptor, CRTAM would be critical to fully delineate the mechanism of action *in vivo*. This can be achieved with a more specific innate lymphoid cell restricted gene, *ncr1*, which encodes the NKp46 receptor. Using a specific *ncr1*-driven CRTAM deletion modality, NK cells could be sorted and re-introduced to a mouse deficient in all lymphoid cells for experimentation. This could also be expanded to assess if there is a specific NK cell subset responsible for the metastatic control as well.

Although NK cells were the focus of this study, it is important to investigate the role of other immune cells in the control of metastasis, either in conjunction with or independent of NK cells. The role of cytotoxic T cells might be particularly important, as sub-populations as these cells also express KLRG1 and CRTAM receptors that recognize ECAD and CADM1 (Henson and Akbar, 2009; Leavy, 2008). We have shown that T cells were dispensable for murine LLC metastasis, but this may be due to the low MHCI expression on this specific cell line. More highly-expressing MHCI tumor models would likely reveal a role for cytotoxic T cells as well.

Melanoma and colon cancer tumor models have shown critical roles for T cells. Further investigation may reveal a T cell-mediated mechanism of metastatic control in these models.

Current and Future Immunotherapies

EMT has been implicated in conferring resistance to both conventional therapies including chemotherapy, radiation therapy, and targeted-therapies such as anti-EGFR small molecules. In addition, EMT based gene-signatures has been shown to predict patient prognosis (Lou et al., 2016; Mak et al., 2016). For example, a 20-gene signature that we derived from an EMT-associated secretory phenotype predicted patient survival in NSCLC (Reka et al., 2014). More recently, primary tumors stratified based on an EMT score showed a strong enrichment for immune check point molecules in mesenchymal-like tumors compared to epithelial-like tumors, including significant upregulation of PD-L1&2, PD-1, and CTLA4 (Mak et al., 2016). This suggests that EMT-based biomarkers can be valuable to select patients who may benefit from immune checkpoint blockade agents and other immunotherapies in cancer. Similarly, engineering chimeric antigen receptor T cells or NK cells to specifically target mesenchymal-like cells may be a feasible metastasis-specific prevention strategy. Cell culture models of cytokine-induced EMT can be valuable tools in this endeavor, particularly in identifying specific targets expressed by mesenchymal-like cells.

Conclusions

In summary, contrasting the prevailing notion that EMT confers only tumor promoting functions in cancer cells, this study strongly suggest that EMT also renders cancer cells more susceptible to NK cell cytotoxicity and contributes to the inefficiency of metastasis (**Figure 6-3**). This novel metastasis-specific immune surveillance mechanism presents a potential window of

opportunity for the prevention of metastasis by boosting NK cell functions. Multiple NK-based therapeutic strategies, both at preclinical and clinical stage, are under development for cancer immunotherapy with promising outcomes (Guillerey et al., 2016; Leidner R, 2016). Approaches that can induce or restore activating ligands or that can neutralize inhibitory ligands or receptors may boost NK cell functions. Analogous to CAR-T cells, these approaches can also aid in the design of "super-NK" cells for cancer immunotherapy.

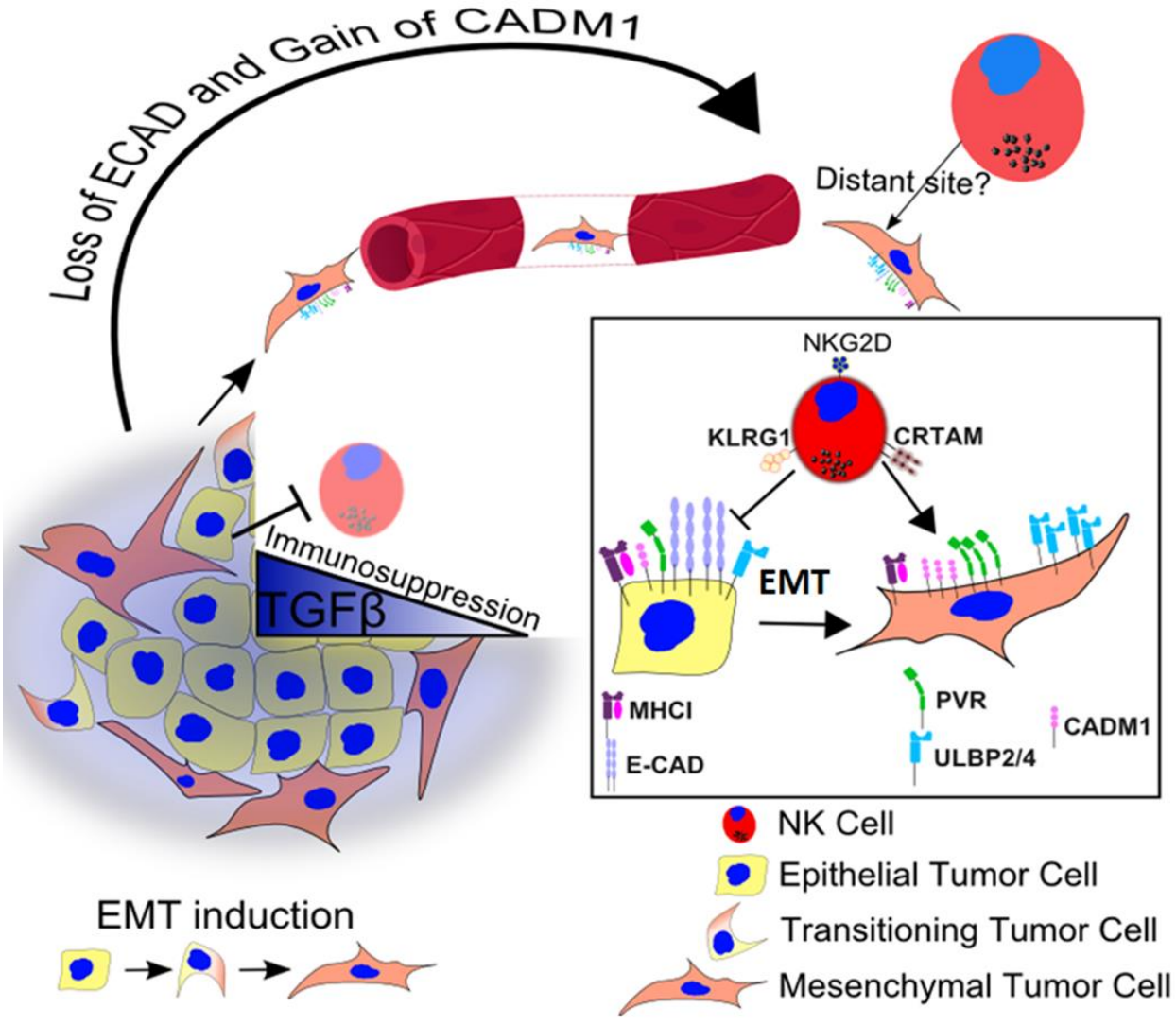

Figure 6-3 Working Model

A general model of our findings depicting the tumor immunosuppression from primary tumors that allows tumor progression and EMT. These transitioned cells escape to the circulation and

transit to the distant site, whereby the reverse MET process takes place. We highlight some of the ligand changes that we have identified with our transcriptomic profiling of A549 cells undergoing TGFβ-induced EMT.

Future Directions and Comments

Experiments that aim to elucidate the role of CADM1 during oncogenesis will be critical to explore. This would involve the use of a combined CADM1-specific deletion in transformed cells *in vivo*. This can be achieved in a similar manner as the PKY mice and merely engineering a conditional CADM1 deletion. Further, this mouse could be used for organ-specific carcinogenesis as well. Given the numerous solid tumor types with reduced *CADM1* expression, it would be fascinating to explore other cancer types to elucidate the immunological mechanisms that may underpin the tumor suppression or patient survival benefit observed. Further, the conserved nature of CADM1 allows for more accurate mechanistic dissection when investigating its role and translating it to a clinical setting.

Delving deeper into the altered immunogenicity of cells undergoing EMT, varying the methodologies used for EMT induction may also reveal the mechanisms of ligand modulation. These experiments could include overexpression of EMT master transcription factors to induce EMT or initiating EMT with TGFβ and sustaining with other cytokines. Both approaches may reveal distinct EMT profiles that could help elucidate targetable alterations in protein profiles. However, overexpression of EMT transcription factors can lead to a mesenchymal locked state and reversal does not occur.

Fully determining the regulatory pathway that cancer cells use to methylate the promotor region of CADM1would also allow for another exploitable target for therapeutic benefit. To this end, utilizing the cell line databases, transcriptomic changes for methyl-transferase alternations

between primary and metastatic sites could be performed to probe this line of inquiry. Accessing matched primary tumor and metastatic site samples from single patients would be critical to pair our hypotheses generated by this project and potential clinical applications.

Finally, and fundamentally, increased exploration into the basic signaling that NK cells utilize to determine responses is needed. Prevailing concepts of positive and negative signaling fails to account for permuted or mixed signaling in determining responses from NK cells. The general consensus that the intracellular signaling domain dictates whether the receptor is activating or inhibition fails to account for many observed phenotypes in which an "inhibitory" receptor determines the activated NK cell population. We must revisit our understanding of how NK cells integrate complex signals. This, assuredly, will entail combining genetic and epigenetic changes upon development and maturation. The "light-switch" concept of cell signaling comes from binary readouts. Altering our modality of reading responses from NK cells or cells in general, may uncover varying degrees of activation or inhibition.

A concept has been posited that NK cells are the first cells to learn how to "count", in the near literal sense of counting a number of something. This idea stems from the recognized balance of activating and inhibitory ligands on target cells that result in cytotoxicity. NK cells assess the number of ligands on a target cell via their cognate receptors and allow both positive and negative signaling to occur. Integrated signaling is likely occurring in these cells and a "decision" is reached on what action or actions will be performed by the cell. A rheostat model of NK signaling seems most appropriate and differs so significantly from the adaptive immune system that relies on single specific cognate epitope recognition to determine responses. The notion of a rheostat model that controls NK cells fits their nature of stochastically-expressed

panoply of germline-encoded receptors. This diversity allows the varied responses of NK cells to malignant or virally-infected cells, for example.

Knowing the full receptor landscape of NK cells that respond or not respond will allow for “smart” cell therapy creation. These cells would retain their ability to “decide” what to and to not target. Deciding what to remove or not has always been at the crux of medicine; medical research has focused on making ever-more specific and refined therapies to do just that. This is a major issue with current chimeric antigen receptor (CAR) therapies; cells have a designated target and kill indiscriminatingly. This may lead to serious and life threatening “off-target” effects on critical organ systems that naturally express the CAR target. Further, loss of the CAR target from the cancer cells is a common occurrence not dissimilar to chemotherapeutic resistance. This, effectively, turns a living and “thinking” cell into a “dumb” drug. Given the inherent nature of NK cells, and how signals are “counted” and integrated, makes them ideal candidates for future immunotherapies to combat cancer.

Chapter 7 Methods

Cell lines and culture conditions

Murine Lewis Lung Cancer (LLC) and (344SQ) and human: A549, NK92mi, H1299, H460, H358, K562, NK92mi and MCF7 cells were grown in 95% air/5% CO_2 at 37° Celsius. Culture media for LLC cells is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.3 mg/mL L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Human cell lines were cultured according to the guidelines listed from the American Type Culture Collection (ATCC). For EMT experiments, cells were grown in 6-well plate in 30–40% confluency (50,000 cells/well) in complete medium. They were serum starved for 24hrs and then treated with TGF-β (5ng/ml) for 72hrs.

Mice

8-week-old male or female C57BL/6J, B6.129S7-*Rag1*^{tm1Mom}/J (RAG1$^{-/-}$) mice were purchased from Jackson Laboratory and housed in SPF conditions. PKY mice were generously gifted from Andrew Rhim. All animal experiments were conducted in accordance with procedures approved by the University Committee on Use and Care of Animals and conformed to the policies and procedures of the Unit for Laboratory Animal Medicine at the University of Michigan.

Tumor Models

Cancer cells were grown in their respective media. Cells were lifted using EDTA and removed with complete media. Cells were spun at 250g in a table top centrifuge and washed 2 times with plain serum free media. Cells were then resuspended in plain media at $10x10^6$ cells per milliliter of media and stored on ice until injection.

Subcutaneous: Cells were loaded into a 1ml syringe fitted with a 26 gauge needle and 100µl of this cell suspension was injected subcutaneously in the right and left dorsal flanks of mice resulting in 2, $1x10^6$ cells injections per mouse. Tumors were measured twice weekly with manual calipers and the modified ellipsoidal volume equation was used $(LxW^2)/2$=Volume to determine tumor sizes. Mice were euthanized upon reaching a $2500mm^3$ tumor volume limit.

Experimental Metastasis: Cells were grown and lifted via EDTA, washed twice in DPBS and resuspended at $5x10^6$ cells per milliliter of DPBS. 200µl of this cell suspension was injected via lateral tail vein of mice. Mice were monitored and after 8 weeks mice were euthanized and lungs were excised for lung module counts.

De Novo: Adenovirus containing CRE-Recombinase was prepared at $2.5x10^7$ viral particles per 50µL of OPTI-MEM media with $CaCl_2$. Mice were anesthetized with a ketamine and xylazine mixture based on bodyweight. Mice were placed in an angled supine position and tracheas were exposed with a small vertical incision in the neck. A syringe fitted with a bent 26 gauge needle was introduced and viral instillation was performed. Incisions were closed with staples and monitored for anesthetic recovery.

Metastatic Burden Quantification

In all models, lungs and primary tumors were excised and fixed with ice-cold 4% paraformaldehyde and overt lung metastases were counted under magnifying glass. Additionally, lungs were cryoprotected in 30% sucrose solution, embedded in OCT, frozen, and sectioned at 10 micron thicknesses on a cryostat (Leica). Some sections were then mounted with Prolong Gold AntiFade with DAPI (Thermofisher, CAT#P36931) and observed on an epiflourescent microscope or sections were stained with hematoxylin and eosin and observed via brightfield microscopy.

Circulating Tumor Cell Capture

Peripheral blood of RAG1$^{-/-}$ mice was isolated upon tumor limits being reached. Red blood cells were lysed and whole blood was subjected to antibody coated chip capture. Chips were coated with anti-EGFR(20μg/ml), anti- EPCAM(10μg/ml), and CD133(10μg/ml). The circulating tumor cells were identified based on their mCherry positivity. This work was performed by Dr. Vasudha Murlidhar in Professor Sunitha Nagrath's laboratory at the University of Michigan.

Cell Line Creation from PKY Mice

Pieces of either a metastatic nodule or lung lobe bearing over tumor nodules were excised from mice. These tissues were then minced in complete DMEM media and collagenase digested for 1 hour at 37 degrees Celsius with gentle agitation. Suspensions were then centrifuged at 250g and suspended in complete media with Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor at 10μmol/L. This suspension was plated onto mitomycin C inactivated swiss 3T3 fibroblast cells to support growth of cancer cells. Using differential trypsinization and passaging of cancer cells onto new fibroblast feeder cells for 4 weeks we ceased addition of ROCK and new fibroblast cells and attained 100% YFP cultures.

siRNA Transfection

CDH1, KLRG1, NKG2D, CADM1 siRNA molecules were purchased from Integrated DNA Technologies. A scrambled sequence from the same company was used as control. Cells at 30–40% confluent (50,000 cells/well) were transfected with siRNA using Lipofectamine 2000 and OptiMEM medium. After 6 hours of transfection, cells were allowed to recover overnight from transfection in RPMI-1640 medium with 10% FBS before inducing EMT or further assessments as indicated. NK92mi cells, 10^6, were transfected in a 5ml round bottom tubes.

Doxycycline Inducible CADM1 Gene Expression

CADM1 cDNA approximately 1362bp was created using these two primers

5’ GGGCGGCCGCCAGGTGCCCGACATGGC 3’ NotI containing N-terminal

5’ AAGGAAAAAAGAATTCCAGTTGGACACCTCATTGAA 3’ EcoRI containing C-Terminal

Annealing temperature 65° Celsius, 35 cycles, 40 second annealing time, 90 second extensions using Promega GoTaq green mastermix, CAT# M7122, primer concentration were 1μM each. Lentiviral pLVX-TRE3g and pLVX-TET3g plasmids, CAT#631187, Clontech, were used to create stable doxycycline-inducible cell lines. pLVX-TRE3g was cut with EcoRI-HF,CAT#R3101S, and NotI-HF, CAT#R3189S, New England Biolabs Inc, restriction enzymes. These same enzymes were used to cut the PCR CADM1 product overnight and were subsequently heat-inactivated and gel-purified in 2% agarose and visualized with ethidium bromide for 1hour at 110V. Bands were excised and purified using Qiagen Gel Extraction Kit, CAT#28704. Vector and insert were combined at 1:3 ratio and ligated overnight at ~16° Celsius using T4 DNA ligase, CAT#M0202T, New England Biolabs Inc.

Ligation reactions were transformed into One Shot STBL3 E.Coli cells, CAT# C737303, ThermoFisher Scientific, via heat shock for 42 seconds and plated on ampicillin 100ug/mL agar plates overnight. Colonies were isolated in 20μl LB media and PCR was performed to initially screen for CADM1 cDNA expression. Positive colonies were then grown overnight in 5mL LB media containing 100μg/ml ampicillin plasmids were purified using QIAprep Spin, CAT#27104, Qiagen.

Purified plasmids were digested with EcoRI and NotI and gel electrophoresed plasmids containing inserts were sequenced using MSCV reverse primer.

5’ CAGCGGGGCTGCTAAAGCGCATGC 3’

Sequencing was performed at the University of Michigan Sequencing Core.

Sequence validated plasmids were then utilized in lentiviral production.

Lentiviral Particles were made at the University of Michigan Vector Core Cancer Center (P30 CA046592)

CRISPR/Cas9-Mediated Deletion

A549 expressing Cas9 cells were purchased from Genecopiea CAT#SL504. Signal guide RNAs were also purchased from Genecopiea.

CADM1, CAT# HCP206321-LvSG03-3b

Non Targeting, CAT# CCPCTR01-LvSG03-3b

SMAD2, CAT# HCP254007-LvSG03-3b

SMAD3, CAT# HCP210960-LvSG03-3b

SMAD4, CAT# HCP210961-LvSG03-3b

TGFβRI, CAT# HCP218051-LvSG03-3b

these bacterial stocks were plated and a single colony was selected for plasmid growth and subsequent virus production. Lentiviral particles containing the specific targeting constructs were then used to infect A549-Cas9 cells. These new cell lines were verified by puromycin selection 1μg/ml and mCherry expression validated by flow cytometry.

Western Immunoblot Analysis

Cells were washed with Dulbecco’s Phosphate Buffered Saline (DPBS), pH 7.4, after treatment and lysed in radio immunoprecipitation assay buffer (RIPA) containing NaF, Na3Vo4 and protease inhibitor. Samples containing 10-20μg of total protein were electrophoresed on SDS-

polyacrylamide gels and transferred onto a polyvinyldifluoride membrane by electro-blotting. Membranes were probed with primary antibodies.

Anti-CADM1 (3E1), CAT# CM004-3, MBL, 1:1000

Anti-CADM1 (polyclonal), CAT# ABT66, EMD Millipore 1:500

Anti-SynCAM (polyclonal), CAT#S4945, Sigma-Aldrich, 1:1000

Anti-ECADherin (36/ECADherin), CAT# 610182, BD Transduction Laboratories 1:1000

Anti-N-Cadherin (32/ECADherin), CAT# 610921, BD Transduction Laboratories 1:1000

Anti-GAPDH-HRP (GAPDH 71.1), CAT# G9295, Sigma-Aldrich 1:10,000

Anti-Vimentin (13.2) CAT# V5255, Sigma-Aldrich, 1:1000

Anti-SMAD2, CAT#5339, Cell Signaling Technology, 1:1000

Anti-SMAD3, CAT#9523, Cell Signaling Technology, 1:1000

Anti-SMAD4, CAT#38454, Cell Signaling Technology, 1:1000

Secondary Antibodies containing horseradish peroxidase(HRP)

Anti-Mouse-HRP (polyclonal), CAT#A9044, Sigma –Aldrich, 1:80,000

Anti-Chicken HRP (polyclonal), CAT#31401, Thermofisher 1:10,000

Anti-Rabbit HRP (polyclonal), CAT#A0545, Sigma-Aldrich, 1:40,000

Immunodepletion

The following antibodies were administered into the intraperitoneal space to deplete NK cells; per mouse: 25µl of stock rabbit polyclonal anti-Asialo-GM1 (ASGM1), cat. no. 986-10001, Wako, diluted to a final volume of 100µl in DPBS. 200µg murine monoclonal anti-NK1.1 (clone:PK136), CAT# BP0036, BioXcell, diluted to a final volume of 100µl in ddH2O. 100µl of undiluted normal rabbit serum (NRS), CAT# 16120, Life Technologies, was used as the control

for ASGM1. 200µg murine monoclonal anti-IgG2A (clone:C1.18.4), CAT# BP0085, BioXcell, diluted to a final volume of 100µl in ddH2O was used as the control for anti-NK1.1.

Flow Cytometry

Cells were collected and spun at 250g and resuspended in FACS buffer(DPBS with 1% BSA) Non-specific antibody binding was blocked with either CD16/32 for mouse or 5% human serum for 10 minutes at room temperature. Cells were spun and washed in FACS buffer. Primary antibody incubations were performed at indicated dilutions for 30 minutes on ice. CD3ε (145-2C11) 1:200, NK1.1(PK136) 1:100, CD45(30-F11) 1:200 BD Biosciences. KLRG1 (13F12F2), 1:100, eBioscience, CD107A (H4A3) 1:100 BioLegend. Cells were analyzed with an Attune Acoustic Flow cytometer (Applied Biosystems, Carlsbad, CA)

Sorting was performed on a FACS ARIA IIIu at the University of Michigan Flow cytometry core.

Analysis was performed with FlowJo version 10, FlowJo, LLC.

Human Natural Killer Cell Isolation

Human NK cells were isolated in an untouched manner using a commercially available MACS NK isolation Kit, Miltenyi Biotec, CAT#130-092-657. This was performed to the manufacturer's specifications. Briefly, donated peripheral blood samples from healthy donors were subjected to ACK red blood lysis and white blood cells were collected by centrifugation. Cells were counted and appropriate amounts of antibody cocktail were added from the NK isolation kit as per the manufacturer's instructions. This cell mixture was subjected to magnetic field and unlabeled NK cells were collected from the elutant.

Cytotoxicity Assay

Target cells were cultured and treated with TGFβ (5ng/mL) in 1% serum media for 3 to 12 days, depending on cancer cell type as previously described. These cells were then lifted with EDTA, washed in DPBS, and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 0.5μM concentration for 10 minutes. The staining was stopped with ice-cold complete media for 5 minutes, spun at 250g, and washed twice. Cells that were already GFP or mCherry positive were not stained and immediately used. These cells were counted and co-cultured for 4 hours with NK92mi killer cells in complete media corresponding to the cancer cells. These reaction tubes were then placed on ice after 4 hours and Propidium Iodide (PI) was added to a final concentration of 100ug/mL or Sytox Blue (Thermofisher, CAT#S34857) and incubated for 5 minutes and then analyzed via flow cytometry. Cells that were labelling dye or fluorophore positive and PI or Sytox Blue positive were considered dead and to determine the NK-specific lysis the spontaneous death from cultures lacking NK cells were subtracted.

Conjugation Assay

NK92mi cells were labeled with CFSE, as described above, and then allowed to co-culture with target cells at a 1:1 ratio for various amounts of time. Target cells were 100% mCherry positive as described from our cell line creation. Cultures tubes were vortexed briefly to disrupt non-specific binding for a more accurate analysis of conjugated cells. Analysis was performed via flow cytometry. Doublet discrimination was not performed as to allow for the analysis of two cells synapsed together. Here, we quantified the amount of conjugated cells over time via a two color quadrant gate. Whereby the green stained NK92mi cells and the mCherry target cells would lie in their respective single positive quadrant gates and the conjugated cells would be double positive.

Trypsinization Assay

A549 cells were induced with TGFβ to undergo EMT or not. These cells were then lifted with EDTA to retain surface proteins and treated with trypsin for 0, 5, or 10 minutes. These cells were then subjected to co-culture with NK92mi cells and cytotoxicity was measured by flow cytometry, as described previously.

Immunocytochemistry/Immunohistochemistry

Cells were grown on glass coverslips, after treatments cells were fixed with ice-cold methanol for 15 minutes at -20 degrees Celsius. Cells were labelled with anti-E-cadherin(36/E-cadherin) conjugated with FITC, BD Transduction Laboratories, or with CADM1(ABT66) EMD millipore with subsequent anti-rabbit Alexa-488 (R37116) secondary. Cells were mounted with Prolong Gold Antifade with DAPI and observed under a Nikon Eclipse Ti epifluorescent microscope. Primary tumor sections were immunolabeled with anti-E-cadherin (36/E-cadherin) conjugated with FITC, BD Transduction Laboratories, or with anti-SynCAM (S4945) Sigma-Aldrich with subsequent anti-rabbit Alexa-488(R37116) secondary.

Statistical Analysis

All statistics were performed in Graphpad 6 from Prism. Violin plots were created in R with ggplot2 package, Version 1.0.143, RStudio, Inc. Heatmaps were generated with Morpheus, (https://software.broadinstitute.org/morpheus/). Metastatic quantifications were analyzed with a Mann Whitney U test. All survival analyses are Kaplan-Meier Survival Curves with Mantel-Cox Log Rank. Multiple group analyses were performed with a one-way or two-way ANOVA with Tukey's post-hoc analysis. All other statistics are unpaired, two-tailed, student's t-tests. Significance is delineated by *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ and mean±SEM is shown.

Study Approval

All animal experiments were approved by the IACUC of the University of Michigan and performed according to NIH guidelines. Blood samples were obtained with written and informed consent under IRBMED #HUM00075841 from the University of Michigan.

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Appendix

NK Cell Presence does not Affect ECAD or CADM1 Expression In vivo

Analysis of ECAD and CADM1 expression in primary A549 tumors from previous depletion experiments (**Figure APP** 1**A-B**) showed no difference with or without NK cell depletion; which is consistent with lack of effect on primary tumor growth kinetics. Together with analysis of ECAD and CADM1 expression in susceptible cell lines (**Figure APP** 2) further suggests that potentially it is the ratio between ECAD and CADM1 that dictates susceptibility to NK-mediated cytotoxicity. Interestingly, CADM1 knockout had no effect on growth or TGFβ-induced EMT (**Figure APP** 3**A-B**). This data indicates that, putatively, CADM1 is relevant to cancer cells in the context of NK cells only.

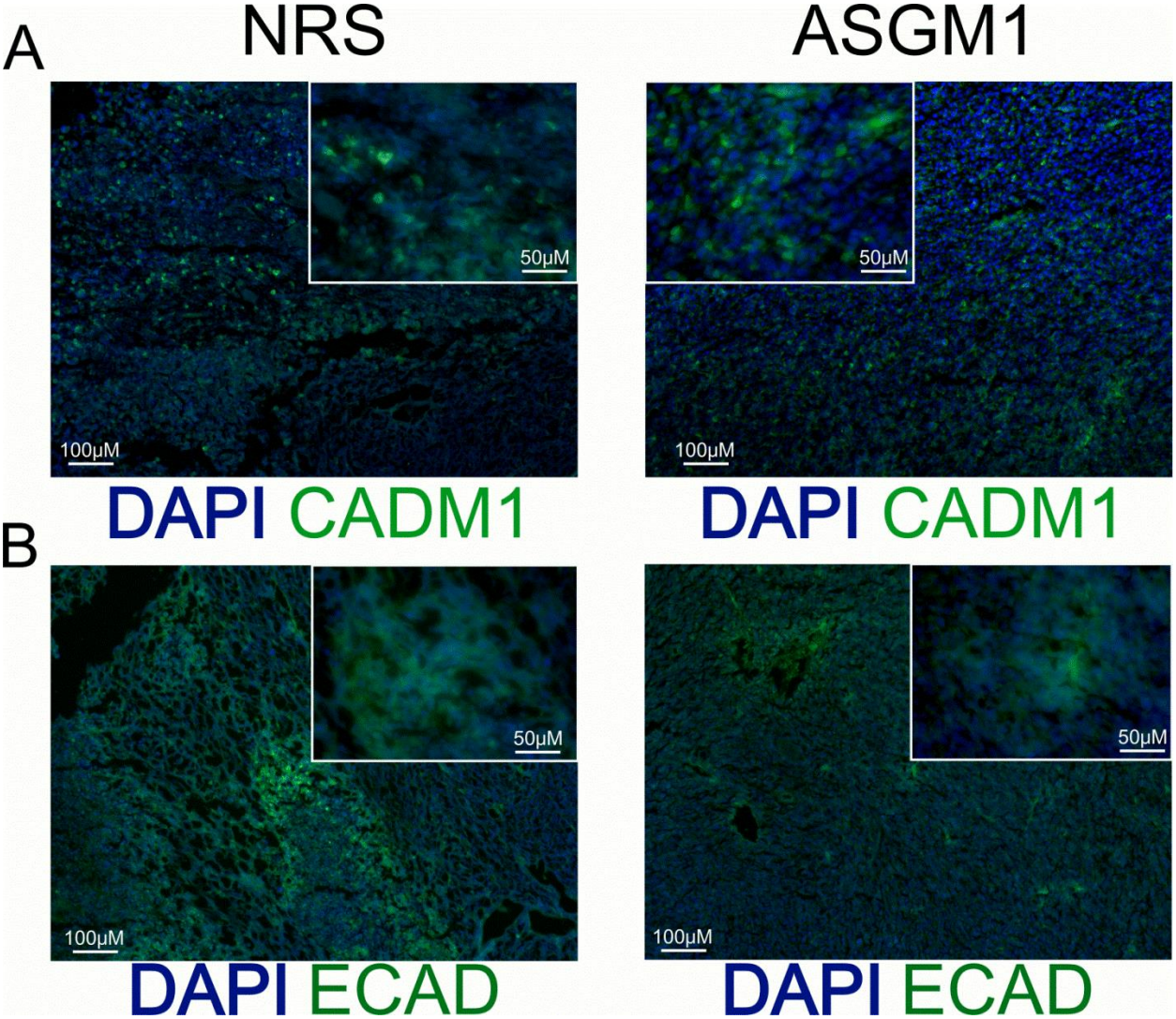

Figure APP 1 CADM1 and ECAD Staining in A549 Tumors

(**A**) A549 tumors grown subcutaneoulsy in RAG1$^{-/-}$ with NK cells depleted (ASGM1) or control (NRS) immunolabeled for CADM1 in green and nuclei were stained with DAPI in blue. Scale bars are 100μm for large images and 50μm for insets.
(**B**) Same tumors were immunolabeled for ECAD in green and nuclei were stained with DAPI in blue. Scale bars are 100μm for large images and 50μm for insets.

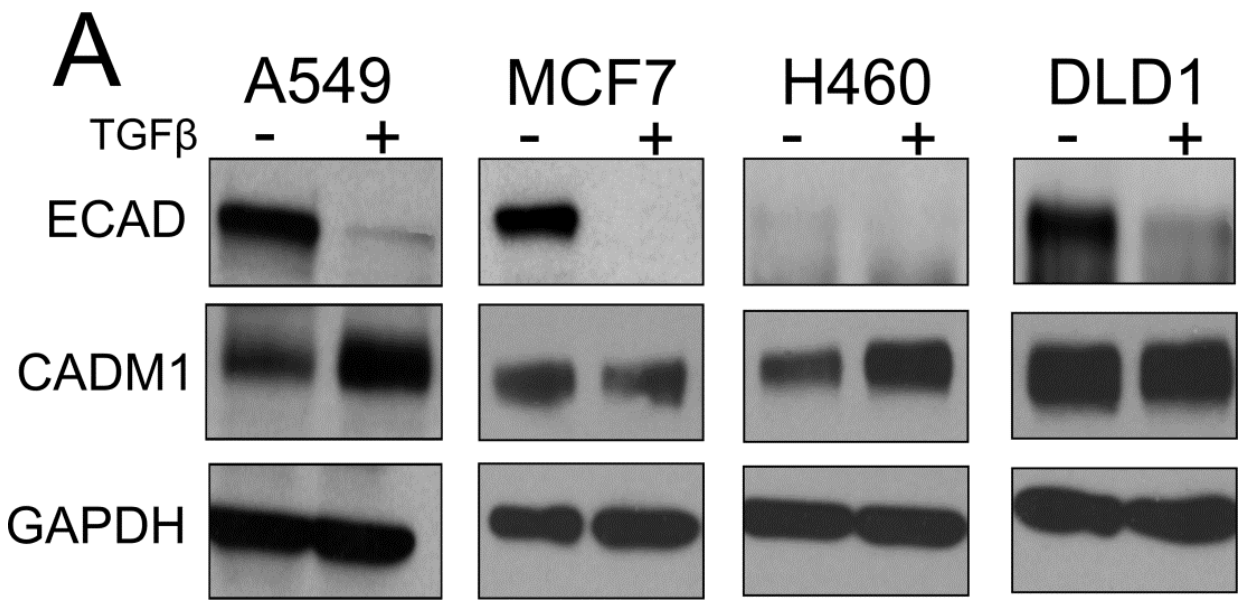

Figure APP 2 CADM1 and ECAD Protein Levels Correlate to EMT-induced Susceptibility

(**A**) Western immunoblot analysis of ECAD, CADM1, and GAPDH in response to TGFβ after 3 days for A549, and 6 days for MCF7, H460, and DLD1. Immunoblots were cropped for clarity of each cell line used.

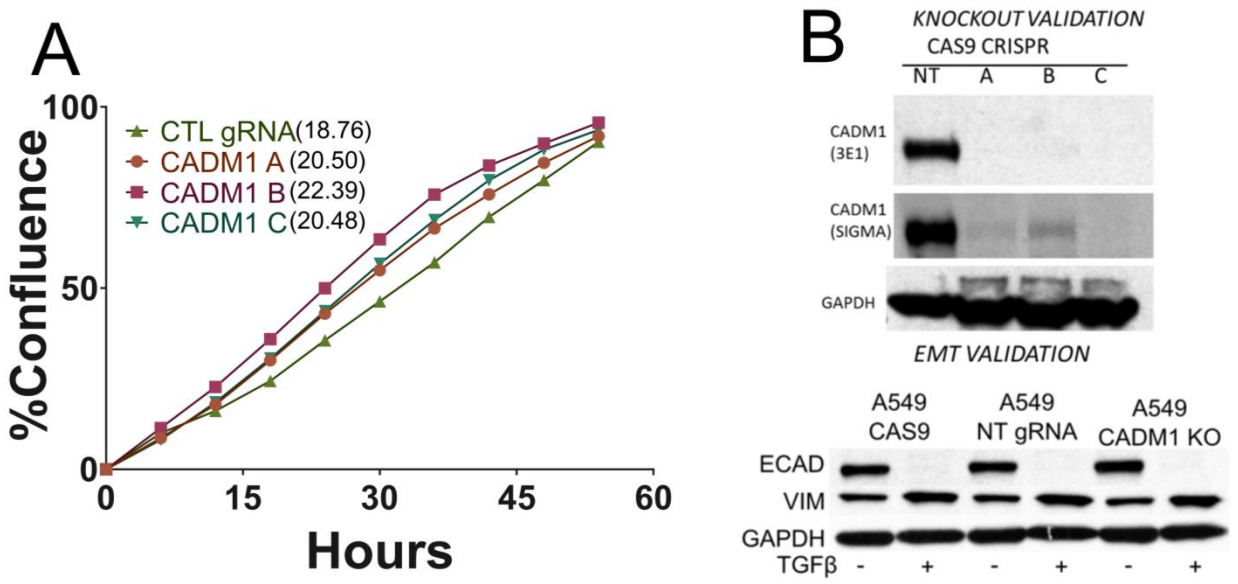

Figure APP 3 CADM1 Deletion Does Not Effect Cell Growth or EMT

(**A**) Confluence percentage as calculated by an Incucyte Imager, EssenBio INC. of A549-CAS9 with indicated signal guide RNAs against CADM1 and a non-targeting (NT) construct. Doubling times were not significantly different, shown in parentheses.
(**B**) Western immunoblot validation of CADM1 deletion utilizing two separate antibodies. 3E1 recognizes the N-Terminal of CADM1 while SIGMA recognizes the C-Terminal domain. Clone C was chosen as our CADM1 KO cell line and TGFβ-induced EMT was validated as seen by ECAD loss and Vimentin (VIM) increases.

CADM1 Expression in Human-derived Cell Lines

To further corroborate our patient cohort data, we investigated a published transcriptomic profile of various human lung cancer cells lines. These lines were then stratified on their anatomical site of origin. We investigated the *CADM1* levels of these cells lines (**Figure APP** 4). We show a significant reduction in *CADM1* when observing cell lines isolated from metastatic sites, pleural effusion or lymph node, when compared to the lung primary tumors. There are many caveats to this analysis especially since matched samples of a patient's primary tumor and metastasis is not present.

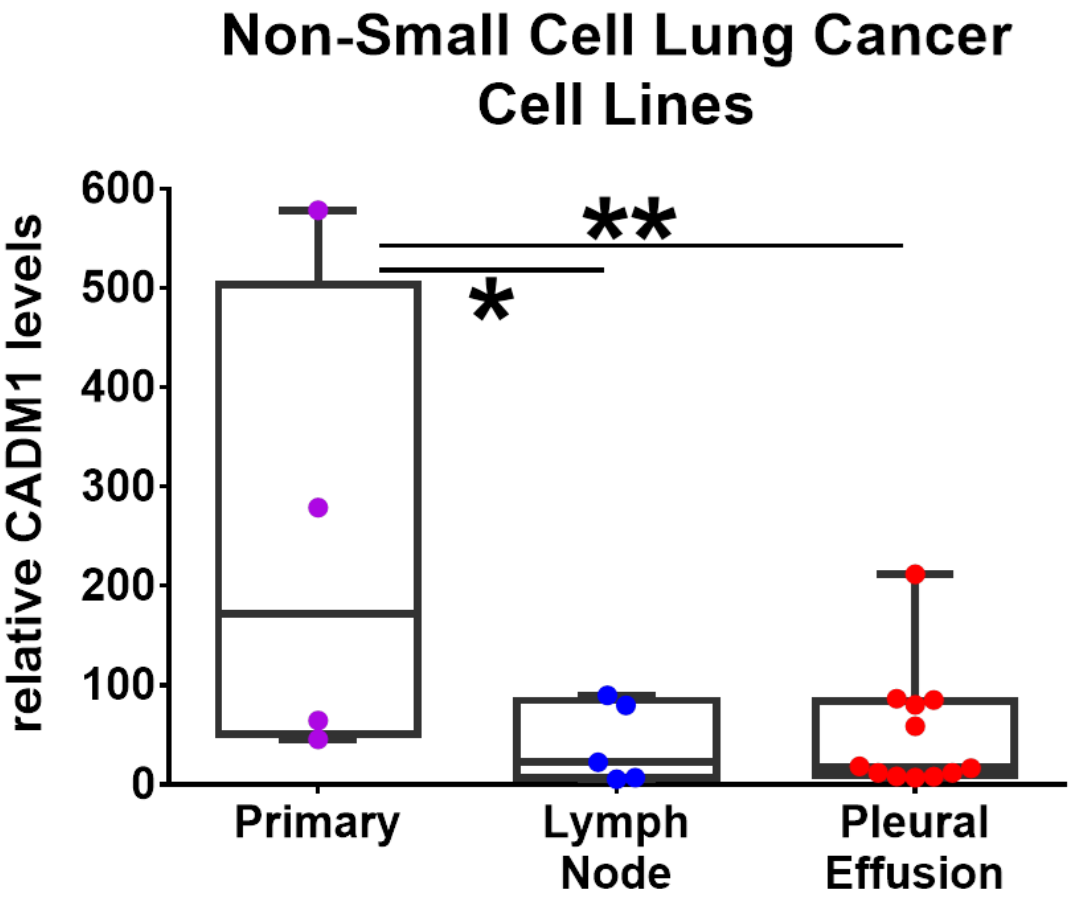

Figure APP 4 *CADM1* Levels from Human Non-Small Lung Cancer Cell Lines

Cancer cell lines derived from various patients (Phelps et al., 1996) and later transcript levels were determined (Zhou et al., 2006). The Zhou et. al database was assessed for *CADM1* levels and the cell lines were cross-referenced to the Phelps, et. al. clinical data that identifies the source of the cell lines. Each dot represents a unique cell line and the isolated site is delineated. Primary meaning lung and the metastatic sites of lymph node or pleural effusion are shown. One way ANOVA with Bonferroni's multiple comparison post-hoc analysis.

Table 2 Human Cell Line Locations and Relative CADM1 Transcript Levels

Primary	H1395(A)	45.51782
	H2347(A)	64.5
	H23(A)	278.9236
	A549(A)	578.4111
Lymph Node	H2009(A)	22.48054
	H1648(A)	6.81196
	H1993(A)	79.54559
	H2087(A)	89.84906
	H1299(A)	5.262238
Pleural Effusion	H2122(A)	7.910377
	Calu-3(A)	18.17012
	H1355(A)	86.37391
	H1437(A)	58.56372
	H1792(A)	85.08834
	H1666(A)	16.42691
	H358(A)	11.81054
	H2126(A)	8.121657
	H460(A2)	80.25198
	Calu-1(A)	6.946154
	H157(A)	11.81104

SMAD2/3/4 Stable Cell Line Creation

Stable cell lines were created in the same manner as CADM1 and TGFβRI. Western immunoblot and EMT blockade allowed us to select which constructs to use. Construct "B" was selected for all constructs they had the maximal deletions of their respective proteins of interest.

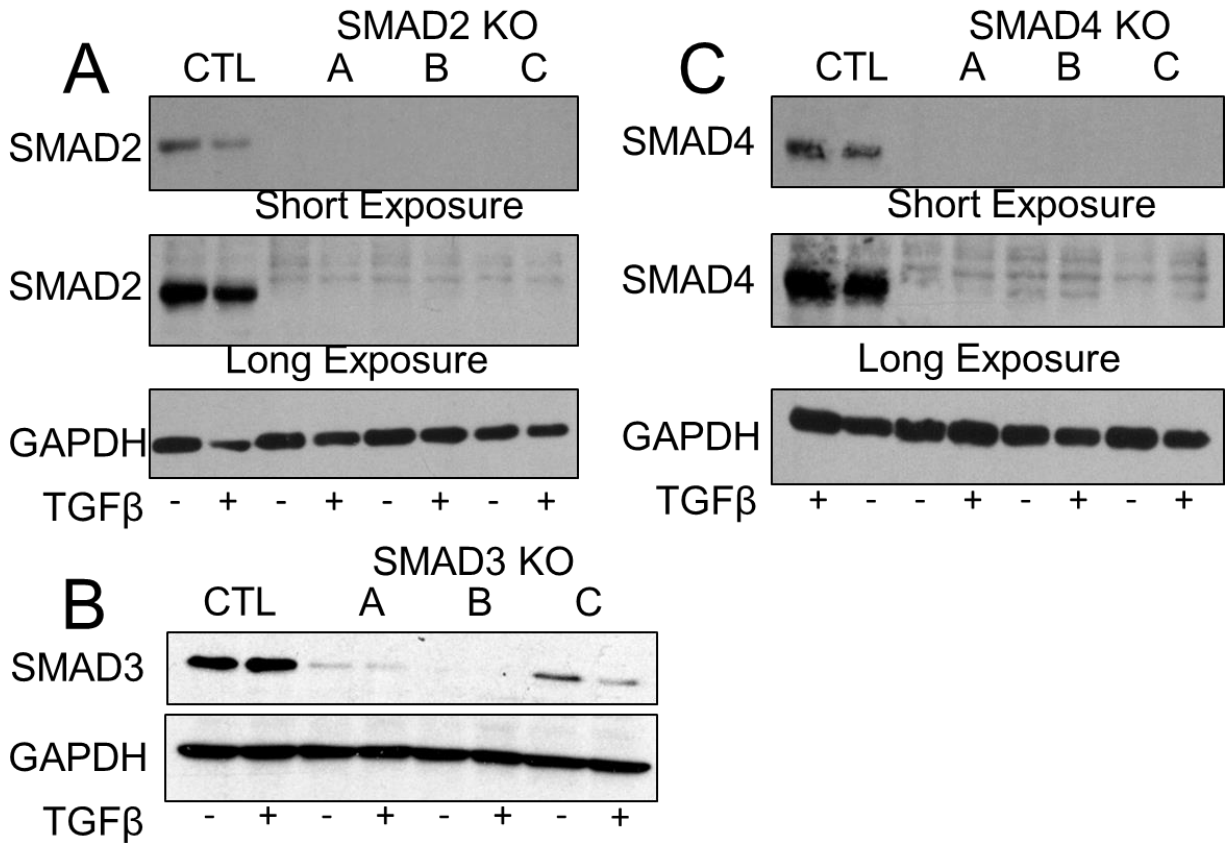

Figure APP 5 Stable CRISPR/Cas9-Mediated Deletion of SMADs 2/3/4 A549 Cell Line

(**A-C**)Western immunoblot analysis of various SMAD2/3/4 knockout constructs with a short and long exposure showing extent of deletion. TGFβ was used to attempt to upregulate protein levels as well for all SMAD proteins.

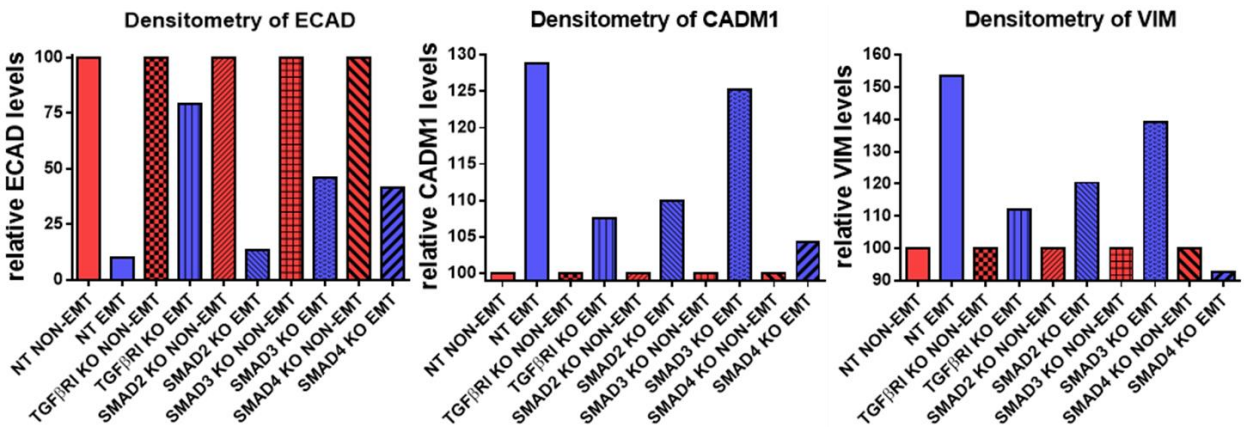

Figure APP 6 Densitometry Analysis of (Figure 5-8)

Relative protein levels were determined by the relative expression of each protein band normalized with their respective GAPDH band.